Laboratory Outlines in BACTERIOLOGY *and* IMMUNOLOGY

NORTON *and* FALK

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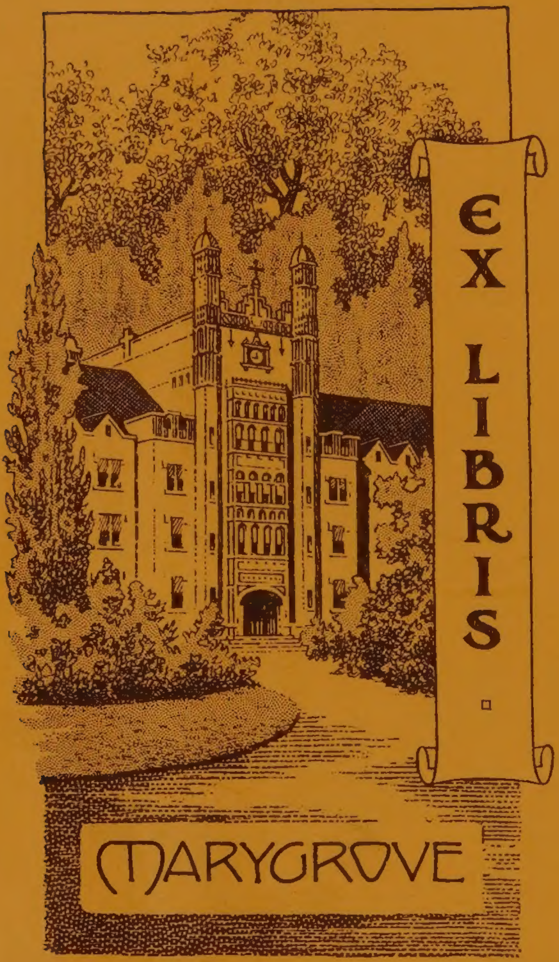
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LABORATORY OUTLINES IN BACTERIOLOGY AND IMMUNOLOGY

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FOREWORD

This manual is presented as an aid to the instructor in arranging courses in bacteriology, serology, and immunology, and to serve the student as a laboratory guide. The time required to complete the experiments is approximately one year, but we have attempted to so arrange the material that it may be used for courses covering much less time.

These outlines can and should be used only under the direction of a competent instructor. We have purposely omitted many technical details concerning which practice varies. For example, no attempt is made to describe the technique of transferring bacterial cultures from tube to tube. There are several ways in which tubes, plugs, and inoculating needles may be manipulated, all of them satisfactory provided contamination is avoided.

The manual is divided into sections which deal with particular methods, with particular groups of bacteria, or with types of immunologic phenomena. These sections necessarily vary in length. In laying out a laboratory course, the instructor should select the sections or portions of sections he desires to use. This selection will depend upon the character of the course offered and the time available for laboratory work as regards hours per week and number of weeks. We have tried to make the manual as flexible as possible. The sections do not have to be assigned in the printed sequence.

A few suggestions for the benefit of the instructor may not be out of place. The media prepared in Section I are not sufficient for the completion of any but a very brief laboratory course. After the student has once learned to prepare media it is unnecessary to ask him to repeat this work. The instructor should supply media as required. Dehydrated products which can be purchased save much time and have proved to be very satisfactory.

Pure cultures of bacteria may be purchased from the American Type Collection, 637 South Wood Street, Chicago.

Descriptions of bacterial cultures are most satisfactorily made on "culture charts." The chart indorsed by the Society of American Bacteriologists is recommended. This may be obtained from Dr. H. J. Conn, Agricultural Experiment Station, Geneva, New York. The University of Chicago Bookstore prints a modification of this chart which the authors have found useful.

The exercises are intended to cover two-hour laboratory periods, but in some experiments more or less time may be required. When the student's work is light, the remainder of the laboratory period may be used for demonstrations and discussions under the leadership of the instructor. Suggestions for these have been made in some of the exercises, but this very important part of laboratory instruction must be left largely to the discretion of the instructor. The serologic and immunologic sections can be used as a basis for many of these demonstrations in courses where time is too short for the student to perform the experiments himself.

Some of the exercises in the serologic section require more than two hours if the student prepares all or part of the materials needed. Such preparations are not necessary in elementary courses. Many of the experiments in immunology cannot be completed in two hours. We have assumed that these exercises will be attempted only by advanced students whose laboratory working hours are not prescribed.

We are indebted to our colleagues who have aided in the construction of the laboratory courses at the University of Chicago. These courses have formed the basis for this guide.

John F. Norton
I. S. Falk

University of Chicago
March, 1926

SECTION I

PREPARATION OF MEDIA

SIX EXERCISES

EXERCISE 1

1. Obtain key to laboratory locker.

2. Check desk equipment with outfit slip or list (see p. 2). Obtain shortage and return signed outfit slip to instructor. Do not accept chipped or broken apparatus.

3. All glassware used in bacteriologic work must be clean. Boiling of Petri dishes, culture tubes, etc., with soap and water will usually remove laboratory dirt. Occasionally glassware, particularly pipettes, must be immersed in a chromic acid cleaning solution. Microscope cover slips are most easily cleaned by treatment with warm dilute nitric acid. No matter what method of cleansing is used, all glassware should be thoroughly rinsed in hot water. Traces of acid or alkali left in Petri dishes, tubes, or pipettes may interfere with tests, particularly with certain serologic reactions.

4. Plug culture tubes with non-absorbent cotton according to method demonstrated by the instructor. A good cotton plug should extend about three-quarters of an inch into the tube, leaving a sufficient amount outside of the tube so that the plug can be easily grasped for removal. The plug should be just tight enough so that the empty tube may be picked up and held suspended by the plug. The plug is not a "stopper," and must be sufficiently loose to allow ready entrance of air but tight enough to act as a dirt-and-bacteria filter. Bake the plugged tubes in a hot-

General Apparatus

Steam autoclave. (Arnold sterilizers may be substituted but are not recommended except for special purposes.)

Hot-air sterilizer
Incubator
Centrifuge
Water bath
Water still

Recommended List of Desk Equipment

(This list contains sufficient apparatus for the exercises in this manual with the exception of some of the serologic experiments. However, it is quite feasible to give a course with considerably less material in the students' desks. The list is suggestive rather than obligatory.)

5 wire baskets
200 culture tubes
8 tin cups for culture tubes
10 large potato tubes
15 serologic tubes
15 fermentation vials
2 funnels: one 15 cm.; one 10 cm. with pinch cock, rubber tube, and glass tube attached
3 graduates, one 500 cc., one 100 cc., one 10 cc.
3 Erlenmeyer flasks, two 500 cc., one 1,000 cc.
1 Stender dish (for cover slips)
2 Syracuse watch glasses
2 1-oz. bottles with corks (for xylol and acetone)
1 medicine dropper
1 electric lamp—40 watt, frosted
4 bottles for staining fluids
1 bottle for balsam
1 magnifier (tripod)
1 pipette can, of sheet iron
3 pipettes, 10 cc., in tenths (Mohr's)
6 pipettes, 1 cc., in tenths (Mohr's)
1 glass stirring rod
1 thermometer, 110° C.
1 sheet-iron box
15 Petri dishes
1 saucepan
1 tripod
1 Bunsen burner and tubing
1 ring stand with 3 rings

In addition the student should have available part, or all, of the following:

50 glass slides
50 cover glasses (No. 1)
50 labels
2 towels
2 inoculating needles
1 test-tube cleaner, small size
1 slide box
3 glass hollow-ground slides, 15 mm.
1 glass pencil
1 pair forceps (small size)
2 pair cover-slip forceps
1 book lens paper
1 bottle immersion oil
1 compound microscope with oil-immersion lens and Abbé substage condenser

air oven for 20 minutes at a temperature between 170° and 200° C. If the temperature is too high there is danger of so charring the plugs as to render them useless. This heating sets the plugs so that they retain their shape when removed but does not sterilize the tubes.

5. Procure laboratory notebooks, culture charts, and gowns or aprons as recommended by the instructor. The care and use of special apparatus will be described as needed for the laboratory work.

EXERCISE 2

1. Place dry Petri dishes in cans or wrap them in heavy wrapping paper and sterilize in a hot-air oven for 1½ hours at a temperature between 170° and 200° C.

2. Place dry pipettes in cans or wrap in paper and sterilize as in item 1. When a pipette is used to measure suspensions of pathogenic organisms a cotton plug should be inserted, before sterilization, in the aspirating end.

3. The following exercises describe the preparation of the more common culture media which it is desirable for the student to prepare. More complicated or special media may be provided. It is now possible to obtain in dehydrated form most of the media required for any instructional course. Media should contain the ingredients necessary for the growth of bacteria and should have a proper acid-alkali reaction. For general purposes, a medium should contain meat extract or meat infusion, peptone, and salt. To these may be added solidifying agents such as gelatin or agar, or special ingredients such as carbohydrates, acid-alkali indicators, chopped meat, blood or blood serum, dyes, etc. The acidity or alkalinity will vary with the type of organism studied but for ordinary purposes it should be not far from the neutral point (P_H = 7.0). Sterile skim milk is a common and useful medium.

4. **Preparation of nutrient broth.**—Weigh saucepan and keep record of weight. Place in pan 1,500 cc. of tap water, 4.5 gm. of meat extract, 15 gm. of peptone, and 7.5 gm. of sodium chloride. Heat until dissolved and boil for 5 minutes. Replace water lost by evaporation. To do this, add original weight of pan, water, and ingredients; place pan containing boiled broth on balance and add enough tap water to bring to original weight. Adjust reaction to P_H 7.2 (±0.1) as follows: with a pipette add 1 cc. of broth to 4 cc. of neutral distilled water in a small test tube or evaporating dish, add 5 drops of phenol red indicator (0.02 per cent alcoholic solution), and add N/20 sodium hydroxide from a burette until the color of the solution matches that of a standard supplied. Calculate and add to the broth the amount of N/1 sodium hydroxide required to make the reaction P_H 7.2. Check results by repeating titrations. Adjust until reaction is correct. Place broth in flasks, plug with cotton, and sterilize in an autoclave for 15 minutes at 15-lb. pressure, according to directions given below.

5. **Notes on the titration of media.**—The acidity or alkalinity of a medium should be determined on the basis of the actual hydrogen ion concentration rather than in terms of titratable acid or alkali. The hydrogen ion concentration is expressed in terms of the P_H value. The P_H value is the logarithm of the reciprocal of the hydrogen ion concentration. Thus, neutral water has a hydrogen ion concentration of 10^{-7}. The P_H value is 7. P_H values decrease as acidity increases and increase with increasing alkalinity. The method described in item 4 gives results sufficiently accurate for most laboratory work. While bacteria require, according to the species studied, certain P_H ranges, divergences of ±0.1 make little or no difference.

Sterilization generally changes the P_H value 0.1 or 0.2 toward the acid side.

Table I contains the names of some indicators and their P_H range.

Color standards may be purchased in the form of colored solutions or as color charts. The latter are the less expensive and give excellent results for general laboratory work. If preferred, the instructor may prepare his own standards (see Clark, *The Determination of Hydrogen Ions* [2d ed.]. Williams & Wilkins, 1922). Sörensen's standards are prepared from 1/15 molal solutions of potassium dihydrogen phosphate (KH_2PO_4) and disodium phosphate ($Na_2HPO_42H_2O$). Care must be taken to insure the purity of the salts. Table II gives the amounts of each solution in a total volume of 20 cc., together with the corresponding P_H value.

TABLE I

Name	Concentration in 95 Per Cent Alcohol; Percentage	P_H Range
Thymol blue	0.04	1.2–2.8
Brom phenol blue	.04	3.0–4.6
Brom cresol green	.04	4.0–5.5
Methyl red	.02	4.4–6.0
Brom cresol purple	.04	5.2–6.8
Brom thymol blue	.04	6.0–7.6
Phenol red	.02	6.8–8.4
Cresol red	.02	7.2–8.8
Thymol blue	0.04	8.0–9.6

6. **Notes on sterilization.**—Most of the media required for the exercises in this manual may be sterilized in a steam autoclave. Place material in autoclave but do not pack closely. Culture tubes should be set in wire baskets, not in tin cups. Flasks should not be so full that there is danger

of wetting the cotton stoppers. Close door of autoclave, but leave valve or pet cock open in order to allow air to escape. Run steam into autoclave until all air is driven out and continue for 10 minutes in order to bring the material to be sterilized to the boiling temperature. Close valve and allow pressure to rise gradually to 15 lb. This pressure is supposed to correspond to a temperature of 120° C.

TABLE II

P_H	M/15 KH_2PO_4	M/15 Na_2HPO_4
5.3	19.5	0.5
5.5	19.2	0.8
5.8	18.4	1.6
6.0	17.5	2.5
6.2	16.1	3.9
6.4	14.5	5.5
6.6	12.3	7.7
6.8	10.0	10.0
7.0	7.8	12.2
7.2	5.7	14.3
7.4	4.0	16.0
7.6	2.6	17.4
7.8	1.6	18.4
8.0	1.0	19.0
8.3	0.5	19.5

It is preferable to use a temperature rather than a pressure indicator, but few autoclaves are so equipped. Allow pressure to remain at 15 lb. for 20 minutes. Shut off steam and close drain valve. Allow pressure to return to zero before opening autoclave.

If no autoclave is available, media can be sterilized in an Arnold sterilizer for 30 minutes on three successive days. Between treatments the media should be allowed to stand at room temperature. The temperature of the steam in the Arnold should be about 98° C. before the time is taken.

Many laboratory workers prefer to sterilize all carbohydrate media in the Arnold. Others use the autoclave for the simpler sugars and the Arnold for the more complex carbohydrates. Filtration through a Berkefeld filter is recommended for precise work.

7. Place 18 gm. of agar in a saucepan, cover with water, and allow to stand until the next exercise.

EXERCISE 3

1. Filter broth through paper, using a pleated filter.

2. **Nutrient agar.**—Pour off excess of water from agar in saucepan. Add 1,200 cc. of filtered broth to agar, boil until agar is dissolved, make up to original volume by adding tap water, adjust reaction to P_H 7.2, and filter through a towel moistened with hot water. (Other methods of clarification, such as filtration through paper or centrifugation, may be used, but the method recommended gives an agar sufficiently clear for most purposes.) Collect the first 200 gm. in a weighed flask and keep hot (see item 3). Tube as much of the remaining filtered agar as is necessary to obtain the following: 25 tubes one-third full (i.e., one-third of the distance from bottom of tube to bottom of cotton plug); 25 tubes one-half full. Any agar not used may be put into a flask.

3. **Dextrose agar.**—To the 200 gm. of melted agar (item 2, l. 8) add 2 gm. of dextrose and enough brom cresol purple indicator to give a distinct purple color. The indicator is made by dissolving 0.5 gm. brom cresol purple in 100 cc. of alcohol. Other indicators, as litmus or Andrade, may be used in place of brom cresol purple. Tube, one-third full.

4. **Broth.**—Prepare 25 tubes of filtered broth, one-third full.

5. Sterilize tubes of nutrient agar, dextrose agar, broth, and agar in flask. The tubes of agar one-third full and the

dextrose agar tubes should be slanted while cooling after removal from the autoclave.

EXERCISE 4

1. Prepare 500 cc. of broth, using the same proportions of ingredients and the same procedure as described in Exercise 2, item 4. (To be used in Exercise 5, item 1.)

2. **Potato medium.**—Place 50 gm. of sliced, peeled potato in 150 cc. of tap water in a flask, plug with cotton stopper, and steam for three-quarters of an hour in the autoclave or Arnold sterilizer. Allow to stand until next exercise.

EXERCISE 5

1. **Sugar broths.**—Filter broth from last exercise. Dissolve 0.5 gm. dextrose in 50 cc. of the broth. Add brom cresol purple or Andrade indicator. Insert an inverted vial in each of 5 plugged culture tubes. Fill tubes not more than one-half full with the dextrose broth. Repeat, using lactose and then saccharose in place of dextrose. It is necessary to use sugars of the highest purity. They can be obtained especially prepared for bacteriologic work. Sterilize sugar broths in autoclave or Arnold. When cool, the air in the vials should have been replaced by liquid. If air bubbles are present, resterilize.

2. Save remainder of broth for next exercise, item 1.

3. **Potato medium.**—Strain potato infusion (from last exercise) through a towel, make up volume to 250 cc. with tap water, add 5 gm. of agar and 5 gm. of dextrose, and heat until dissolved. Replace water lost by evaporation, prepare 15 tubes, one-third full, and sterilize. Slant while cooling.

EXERCISE 6

1. **Gelatin.**—Heat to boiling 250 cc. of filtered broth saved from last exercise, add 40 gm. of gelatin, and boil until dissolved. Replace water lost by evaporation, adjust

reaction to P_H 7.2, and cool to 60° C. Dissolve the white of 1 egg or 3 gm. of powdered egg albumin in a little cold water and mix with gelatin broth. Heat slowly, protecting bottom of pan with asbestos, until the egg albumin coagulates and forms a solid film on the surface of the broth. Filter, taking care not to break film, tube, one-third full, and sterilize.

2. **Milk.**—To 250 cc. of skim milk add sufficient brom cresol purple indicator or litmus solution to give a distinct color. Skim milk prepared from milk powder may be used. Tube 25 tubes, one-third full, and sterilize. Some indicators are decolorized during sterilization but the original color returns after the milk is cool and there has been time for reabsorption of oxygen.

SECTION II

MICROSCOPIC EXAMINATIONS

TWO EXERCISES

EXERCISE 1

1. **Demonstration of the compound microscope.**—The instructor will describe the essential features of the microscope and will illustrate how to clean oculars, objectives, substage condensers, etc., and how to manipulate the various parts. Students should read some elementary book on the microscope. The small volume by S. H. Gage (*The Microscope* [14th ed., 1925]. Ithaca, N.Y.: Comstock Publishing Co.) is especially useful.

2. **Examination of living micro-organisms in hanging-drop preparations.**—Obtain material from the following sources and record observations on the morphology, motility, and Brownian movement of the micro-organisms: tartar from teeth, yeast cake, incubated hay infusion, and suspension of fecal matter. With a match-stick or toothpick draw a thin ring of vaseline around the concavity of a hollow-ground slide. Place a small drop of the material to be examined on the center of a clean cover glass. Invert the vaselined slide over the cover glass with the concavity over the drop. Press down gently. Invert and examine the hanging drop, first with a low- and then with a high-power objective. When examining living micro-organisms it is necessary to reduce the illumination.

EXERCISE 2

1. **India-ink preparation.**—(This method is especially useful for the observation of spirilla and spirochetes.)

Scrape a little tartar from the teeth and suspend in a small volume of water or 0.8 per cent salt solution (0.5–1.0 cc.). Place a drop on a slide; add a drop of drawing ink with a match-stick and mix. Examine by one or both of the following methods: (*a*) Hanging drop: Use the method described in Exercise 1. (*b*) Film method: Place a drop of the mixed material on a clean slide, near one end. Touch the drop with the end of another slide. After the drop has spread along the line of contact, draw the second slide across the length of the first, spreading the material in a thin film. Allow to dry at room temperature. Examine under the microscope with high power dry and with oil-immersion objectives. The organisms will stand out, white or gray, against a mottled-black background.

2. **Stained preparations.**—The morphology of bacteria and other micro-organisms is most effectively studied in stained preparations. The general technique is to smear a suspension of the organisms over a clean cover slip or a small portion of a clean slide, permit the smear to dry at room temperature, fix it to the glass by passing it through a flame two or three times (quickly, so as not to burn or scorch the organisms), and stain the film by one of several methods as described below. After staining, the film is washed in running water, blotted dry, and examined. If it is desired to preserve the slide, place a small drop of balsam over the film, press the cover glass down gently to force out the excess of balsam, and set aside in a warm place to dry. The expressed balsam may then be removed with a bit of cloth or lens paper which has been moistened with xylol (xylene).

If the smears are prepared on cover slips and these are mounted with balsam on slides, neater preparations result for a permanent collection. The technique for staining films on cover slips instead of slides is, however, more precarious and more time consuming. Hence it is recommended that slides be used for all ordinary class work.

The most important of the simple laboratory stains are (1) methylene blue, (2) gentian violet, and (3) fuchsin. *Methylene blue* is satisfactory for a quick preparation when the object is merely to have a stained film for microscopic examination. *Gentian violet*[1] is particularly useful in staining by Gram's method. *Fuchsin* is especially valuable for its unusual penetrating power in staining bacterial cells and spores, so-called "acid fast" bacteria, etc.

The staining solutions can be easily prepared from stock solutions which should always be on hand. The stock solutions are prepared by saturating 95 per cent alcohol with the dye. For use, a little should be filtered through paper and the filtrate diluted with nine or ten times its volume of distilled water.

The penetrating power of these stains may be increased by moderate heating—passing the slide on which the stain has been placed through a flame until a perceptible cloud of vapor rises.

3. **Gram stain.**—Staining by Gram's method has a twofold purpose: (1) to bring out the morphology of the microorganisms; (2) to distinguish certain organisms from others by difference in reaction to the treatment. The basis of the method is that after staining and treating with a suitable iodine solution, some organisms are quickly decolorized by 95 per cent alcohol; others lose the dye only after long treatment with this solvent. The procedure is as follows: Make thin smear of material on slide. Dry in air and fix with moderate heating. Stain 1 minute with aniline gentian (or crystal) violet solution. Pour off the stain. Treat with Lugol's iodine solution for 1 minute. Decolor-

---

[1] Gentian violet is a mixture of dyes. Crystal violet is the most important constituent and may be used instead.

ize with 95 per cent alcohol for 30 seconds. Agitate gently. Counterstain for about 10 seconds with safranin. Wash with water, dry, and examine under microscope.

Note.—Do not wash between steps.

ANILINE GENTIAN VIOLET

Gentian (or crystal) violet (saturated alcoholic solution)	6 cc.
Alcohol (95 per cent)	5 cc.
Aniline water (98 cc. water shaken with 2 cc. aniline oil and filtered through paper)	50 cc.

LUGOL'S IODINE SOLUTION

Iodine	1 gm.
Potassium iodide	2 gm.
Water	300 cc.

SAFRANIN

Saturated alcoholic solution	10 cc.
Distilled water	100 cc.

When stained by Gram's method, certain bacteria do not lose the aniline gentian violet stain and, when examined under the microscope, are found to be colored a deep violet. Others characteristically are decolorized by the alcohol and are found to have been stained a pale pink by the safranin used as a counterstain. Those which have retained the aniline gentian violet are said to be *Gram positive;* those which have lost the violet and have taken the safranin are said to be *Gram negative.*

4. Stain with methylene blue and by Gram's method films prepared from the following materials: (*a*) tartar from teeth, (*b*) spoiled fruit juice, (*c*) sour milk, and (*d*) meat spoiled in water or peptone solution.

Examine under the microscope, first with the high dry and then with the oil-immersion objective.

5. **Impression stains.**—The instructor will provide agar plates which have been exposed to contamination from the air and incubated. Press a cover slip gently on a colony of bacteria, remove without breaking the colony, fix to the cover slip by moderate heating whatever has adhered, stain with methylene blue, wash, dry, mount on a slide, and examine under the microscope.

6. Examine and present a written report upon the morphology of Gram-stained preparations of pure cultures provided by the instructor. Describe: (1) general size, (2) shape, (3) characteristic grouping, and (4) whether Gram positive or negative.

SECTION III

TYPICAL PURE-CULTURE METHODS AND REACTIONS

SIX EXERCISES

EXERCISE 1

1. Make transfers, according to the method demonstrated by the instructor, of the following bacterial organisms: *Bacillus subtilis, Sarcina lutea, Proteus vulgaris, Staphylococcus aureus.* Transfer to agar slants, broth, milk, dextrose agar (slant and stab in butt), potato, and gelatin (stab). Inoculate from the *Proteus vulgaris* culture to tubes containing, respectively, dextrose, lactose, and saccharose broths.

Place the tubes inoculated with *Sarcina lutea* in the desk or locker. Put the others in the 37° C. incubator.

NOTE.—In order to reduce the amount of cultural work for each student, it is often advisable to allow students to work in pairs. In this case each pair should have between them a complete set of cultures, but each student should have his own notebook and set of stained preparations.

2. Not all the exercises will require a 2-hour period. This will leave time for discussions and demonstrations.

EXERCISE 2

1. Make descriptions on culture charts of all cultures inoculated in Exercise 1. Use a separate chart for each organism. Record acid and gas formation—positive or negative—in the sugar broth tubes. To determine the presence or absence of gelatin liquefaction, place the inoculated gelatin tubes on the ice for ½ hour. If no lique-

faction has taken place the tube will completely solidify. Use this method in all gelatin cultural work throughout the course.

2. Prepare gentian violet and Gram stains of each of the four organisms from the agar slant cultures. Make smears directly on slides. If permanent preparations are desired the stained smear may be covered with a glass cover slip on which has been placed a small drop of Canada balsam. Observe stains under the microscope and record staining results and morphology on culture charts.

3. Make hanging drop preparations of each of the four organisms from the agar slant or broth cultures. Observe motility under the microscope and record observations on the culture charts. Hanging drop preparations contain live organisms and should be boiled in soap and water immediately after examination.

EXERCISE 3

1. Make 48-hour descriptions of cultures inoculated in Exercise 1. Record changes only. If no change is apparent, record "no change." If there is no evidence of gelatin liquefaction, repeat observation after five days. Retain all cultures until the work in this section is completed.

2. **Plating of mixed cultures.**—Transfer 1 loop of a broth culture of *Proteus vulgaris* to a tube of sterile broth. Transfer 1 loop of a broth culture of *Staphylococcus aureus* to the broth tube just inoculated with *Proteus vulgaris*. Mix by rotating the tube, held upright, between the palms of the hands. Make agar plates from this mixture according to two methods: (*a*) Melt 3 one-half-full tubes of agar. Cool to 45° C. Transfer 2 loops of the broth containing a mixture of the proteus and staphylococcus organisms to one of the cooled melted-agar tubes. Mix well. Transfer 2 loops from this agar tube to a second, mix, and repeat to

the third agar tube. Pour the contents of each tube into a separate sterile Petri dish. Allow to harden, keeping the dish as flat as possible, invert, and place in the incubator for 48 hours. (*b*) Melt 2 one-half-full agar tubes. Pour each into a sterile Petri dish and allow to harden. Divide each plate in halves by a glass-pencil line drawn on the bottom. Place 2 loops of the proteus-staphylococcus broth mixture on the surface of the agar of 1 plate and streak as demonstrated by the instructor, taking care to keep the material on one half of the plate. Without flaming the needle, streak the other half of the same plate. Repeat inoculation on the second plate, using 1 loop of culture mixture. Invert plates and place in incubator for 48 hours.

3. Make agar slant cultures of the following organisms: *Erythrobacillus prodigiosus*, *Pseudomonas pyocyanea*, and *Spirillum rubrum*. Make dextrose agar slant culture of *Streptococcus lacticus*. Place slant of *Erythrobacillus prodigiosus* in desk or locker. Incubate the others.

EXERCISE 4

1. Prepare gentian violet and Gram stains from agar slant cultures inoculated in Exercise 3, item 3. Examine.
2. Determine motility of these organisms by means of hanging drop preparations.
3. Make transfers from agar slants of *Erythrobacillus prodigiosus*, *Pseudomonas pyocyanea*, and *Spirillum rubrum* to dextrose agar (slant and stab), broth, milk, potato, and gelatin. Transfer *Streptococcus lacticus* to milk and gelatin.

EXERCISE 5

1. Forty-eight-hour descriptions of agar slant cultures and 24-hour descriptions of subcultures (Exercise 4, item 3).
2. Describe, using culture charts, colonies of *Proteus vulgaris* and *Staphylococcus aureus* from plated mixture (Exercise 3, item 2).

3. Transfer, with inoculating needle, 1 colony of each kind from each plate (item 2) to an agar slant. Incubate.

EXERCISE 6

1. Forty-eight-hour descriptions of cultures made in Exercise 4, item 3.

2. Examine agar slants prepared in Exercise 5, item 3. Make gentian violet stains from each slant and record morphology and whether culture appears to be pure or mixed.

3. **Spore stain of *Bacillus subtilis*.**—Möller's method. Prepare smear as usual. Cover with chloroform for 2 minutes. Allow to dry in air and cover with a 5 per cent solution of chromic acid for 2 minutes. Wash in water. Cover with carbolfuchsin[1] and heat for 5 minutes over a small flame or a water bath. Do not allow the dye to dry on the slide. Decolorize with 1 per cent sulphuric acid for 30 seconds, wash in water, and counterstain with methylene blue for 1 minute. Wash in water, dry between filter papers, and examine.

4. Discussion or demonstration.

5. If Section IV is to follow this exercise, make broth cultures of *Staphylococcus aureus* and *Bacillus subtilis* from agar slants. Inoculate a broth tube from a stock culture of *Bacterium paratyphosum B*. Incubate the inoculated broth tubes.

6. All cultures, with the exception of those on which it is desired to make seven-day observations, may be discarded. All cultures should be autoclaved before being destroyed. Do not throw melted agar into the sink. Culture tubes and Petri dishes should be washed immediately after emptying, as dried agar is often difficult to remove.

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[1] Basic fuchsin, 1 part; alcohol, 10 parts; 5 per cent carbolic acid solution in water, 100 parts.

SECTION IV

ACTION OF HEAT AND DISINFECTANTS ON BACTERIA

THREE EXERCISES

EXERCISE 1

1. **Action of heat on bacteria.**—Use broth cultures inoculated at previous exercise (see Sec. III, Exercise 6, item 5). Make stains of broth cultures of *Staphylococcus aureus*, *Bacterium paratyphosum B*, and *Bacillus subtilis* to determine purity. Make fresh transfers to broth of each of these organisms for use tomorrow and incubate. With sterile pipettes, add 0.2 cc. of broth cultures of each of the three organisms to separate sterile broth tubes. Prepare a duplicate set. Place one set in a water bath at 60° C. and the other in a water bath at 85° C. After 15, 30, and 60 minutes transfer 1 loop from each tube to a tube of sterile broth. Incubate these to determine viability of cultures.

EXERCISE 2

1. Examine broth cultures containing transfers of heated suspensions and record presence or absence of growth. Replace in incubator.

2. **Action of disinfectants on bacteria.**—The instructor should determine which disinfecting substances are to be used. Mercuric chloride, phenol, and hydrogen peroxide are recommended since these differ greatly in their germicidal power. Since each student will be unable to study many dilutions of these disinfectants, it is suggested that the experiment be performed by the class as a whole, each

member being assigned certain dilutions for study. The following are recommended: mercuric chloride—dilutions of 1/1,000, 1/5,000, and 1/10,000; phenol—dilutions of 1/20, 1/100, and 1/500; hydrogen peroxide—commercial preparation (3 per cent) undiluted, 1/10, and 1/50. Each student should be assigned three dilutions, not necessarily of the same disinfectant. To these tubes add 0.2 cc. of broth culture of organisms, using 1 tube for each organism. Immerse tubes in water at 20° C. After 5, 15, and 30 minutes, transfer 1 loop to tubes of sterile broth as in previous exercise. Incubate transfers.

EXERCISE 3

1. Examine broth cultures (Exercise 2, item 1) and record final results, i.e., growth as positive, or negative, from action of heat on bacteria.

2. Examine tubes (broth transfers from Exercise 2, item 2) and record results from action of disinfectants on bacteria.

3. General discussion, under leadership of instructor, of results obtained in this section, and of the resistance of bacteria to external influences.

SECTION V

THE COLON-TYPHOID GROUP

ELEVEN EXERCISES

EXERCISE 1

1. Transfer *Bacterium coli*, *Bacterium aerogenes*, and *Bacterium cloacae* to agar slants.

2. **Prepare Endo medium.**—Dissolve 3 gm. meat extract, 5 gm. peptone, and 10 gm. agar in 500 cc. boiling water. Replace water lost by evaporation and adjust reaction to P_H 7.8. To the adjusted medium add 2 cc. of 10 per cent anhydrous sodium carbonate solution. Filter through a wet towel, collecting the filtrate in flasks, two to contain 200 cc. each and one 100 cc. Sterilize. Dissolve 10 gm. of lactose in 50 cc. of distilled water and sterilize. The other ingredients of this medium are a saturated alcoholic solution of basic fuchsin (approximately a 10 per cent solution) and a 20 per cent solution of anhydrous sodium sulphite in water. The latter should be freshly prepared at the time the medium is used. Directions for combining these ingredients of Endo medium are found in Exercise 5, item 2.

This exercise may be devoted largely to discussion or demonstration work.

EXERCISE 2

1. Descriptions of agar slants inoculated in Exercise 1.

2. Prepare and examine hanging drops and Gram stains of the three organisms.

3. Make transfers of each of the three organisms to broth, milk, gelatin, dextrose agar, dextrose, lactose and

saccharose broths, and Russell's medium. Russell's medium should be supplied by the instructor. It consists of nutrient agar to which is added 0.1 per cent of dextrose, 1 per cent of lactose, and an indicator—Andrade or brom cresol purple is recommended. Both slant and stab inoculations should be made on this medium.

EXERCISE 3

1. Descriptions of all cultures inoculated in Exercise 2. Note both acid and gas formation on all the media containing sugars. Record results of both slant and stab inoculations in the Russell's medium.

2. Transfer the following organisms from stock cultures to agar slants: *Bacterium paratyphosum A*, *Bacterium paratyphosum B*, *Bacterium enteritidis*, *Bacterium typhosum*, and *Bacterium dysenteriae* (two strains—Shiga and Flexner).

NOTE.—For general courses, it is recommended that the dysentery strains be omitted.

3. Demonstration.

EXERCISE 4

1. Forty-eight-hour descriptions of cultures prepared in Exercise 2, item 3.

2. Descriptions of agar slant cultures inoculated in previous exercise.

3. Prepare and examine hanging drops and Gram stains from cultures described in item 2.

4. Make transfers of each of the organisms to broth, milk, gelatin, dextrose agar, dextrose, lactose and saccharose broths, Russell's medium, and lead acetate medium. Transfer the dysentery strains also to mannite broth.

NOTE.—The sugar broths should be prepared for the student since his original supply may be exhausted.

Lead acetate medium should be freshly prepared by adding 2 drops

(0.1 cc.) of a 10 per cent lead acetate solution in sterilized water to a melted, cooled tube of agar, and allowing to harden. The medium should be tested before being given to the class since some peptones do not give a strong reaction. Witte's and Armour's peptones have been found to be satisfactory.

EXERCISE 5

1. Descriptions of subcultures inoculated in Exercise 4, item 4.

2. **Prepare Endo plates.**—Melt 200 cc. of Endo agar as prepared in Exercise 1, add 10 cc. of sterile 20 per cent lactose solution, 1 cc. of 20 per cent sodium sulphite solution and 1 cc. of saturated alcoholic fuchsin. Mix thoroughly and pour into sterile Petri dishes. Allow to harden. Not less than 10 plates should be prepared.

3. **Prepare 10 eosin methylene blue agar plates.**—Eosin methylene blue agar is prepared as follows (Levine's formula): add 10 gm. of peptone, 2 gm. of dipotassium phosphate (K_2HPO_4), and 15 gm. of agar to 1 liter of distilled water. Boil until all ingredients are dissolved and replace water lost by evaporation. It is not necessary to adjust the reaction. Sterilize in flasks containing 100 cc. each. While still hot, or after cooling and remelting, add the following ingredients to each 100 cc. of agar: 5 cc. sterile 20 per cent lactose solution, 2 cc. of 2 per cent aqueous solution of eosin (yellowish), and 2 cc. of 0.5 per cent aqueous solution of methylene blue. Mix thoroughly and pour into Petri dishes. The medium decolorizes when hot, but the color returns on cooling.

4. Discussion of reactions of organisms of the colon-typhoid group.

EXERCISE 6

1. Forty-eight-hour descriptions of subcultures from Exercise 4, item 4. Milk cultures should be examined again after seven days since some of the organisms in this group produce characteristic changes in milk only after seven

to ten days' incubation. In certain instances even a longer period of incubation is necessary.

2. **Inoculation of Endo plates.**—Make streak inoculations from agar slants or broth cultures of the following organisms: *Bacterium coli, Bacterium aerogenes, Bacterium paratyphosum A, Bacterium paratyphosum B, Bacterium typhosum,* and *Bacterium dysenteriae.*

3. Plate the following mixtures according to the method described in Section III, Exercise 3, item 2: (*a*) *Bacterium coli* and *Bacterium paratyphosum B;* (*b*) *Bacterium aerogenes* and *Bacterium paratyphosum A;* (*c*) *Bacterium coli* and *Bacterium typhosum.*

4. Inoculation of eosin methylene blue plates. Use the procedure as described in item 2, but use only mixture (*c*).

EXERCISE 7

1. Examine and describe appearance of growth on Endo and eosin methylene blue plates inoculated with pure cultures (Exercise 6).

2. Compare the pure culture colonies with those on the plates containing mixtures and record observations.

3. Transfer, to Russell's medium, 1 colony of each type from each set of Endo and eosin methylene blue plates (8 colonies in all).

4. Prepare agar slant cultures of *Bacterium typhosum, Bacterium paratyphosum A, Bacterium paratyphosum B, Bacterium coli,* and *Proteus vulgaris.* This is in preparation for agglutination tests in the next exercise. When the simpler tests (Sec. XI, Exercise 3) are to be used, make transfer of organism designated by the instructor.

5. Demonstration.

EXERCISE 8

1. Observe tubes of Russell's medium inoculated in Exercise 7, item 3. The reactions obtained will determine whether or not the mixed culture has been separated.

2. Inoculate agar slants from cultures made in Exercise 7, item 4.

3. Macroscopic agglutination tests (see Sec. XI, Exercises 3 or 4).

NOTE.—When the simpler tests are used, Exercises 8 and 9 may be combined (see Sec. XI, Exercise 3, items 1–3).

EXERCISE 9

1. Inoculate agar slants from cultures made in Exercise 8, item 2.

2. Observe and record results from macroscopic agglutination tests.

3. Discussion of the agglutination reaction.

EXERCISE 10

1. Perform Widal tests on unknown samples of dried blood.

2. Make macroscopic agglutination tests on unknown sera and cultures supplied by instructor.

3. Inoculate an agar slant with *Bacterium coli* (for Exercise 11).

EXERCISE 11

1. Preparation of a vaccine (see Sec. XI, Exercise 1).

SECTION VI

IDENTIFICATION OF UNKNOWN ORGANISMS

THREE TO FIVE EXERCISES

From three to five exercises can be spent profitably in isolating and identifying bacteria, both from pure and mixed cultures. "Unknowns" should be prepared by the instructor and given to the student without any directions as to how to handle them. While the instructor should be ready to give advice, this should be done cautiously and on the student's initiative.

The bacteria studied in Sections III and V form the basis for these "unknowns," but other organisms of the colon-typhoid group may well be included. "Immune" sera may also be used for identification. The authors have found in their own classes that it is undesirable to give a student a mixture containing more than three different organisms. *Bacillus subtilis* should not be included in unknown mixtures because it will overgrow most other bacteria. Yeasts may well be included in "unknowns," but only morphologic identification should be required.

SECTION VII

BACTERIA FROM THE RESPIRATORY TRACT

FIVE EXERCISES

EXERCISE 1

1. **Preparation of blood agar.**—Obtain 25 tubes of veal infusion agar (P_H 7.4) from the instructor. If veal infusion agar is not available, meat extract agar may be substituted, but the latter does not give as good results with some of the organisms in this group. Melt the tubes in a bath of boiling water and cool to 50°–60° C. While the agar is still fluid, add to each tube 0.5 cc. of blood. Blood from any source may be used. Horse or sheep blood is usually the most convenient to obtain. The blood must be collected aseptically and defibrinated or citrated to prevent clotting. The blood is most easily added to the tubes of agar from a sterile burette, but sterile pipettes may be used. After adding the blood, mix immediately by rotating the tubes between the palms of the hands. While the blood agar is still melted, make 6 blood agar plates by pouring into sterile Petri dishes. Allow all but 2 of the remaining tubes to cool in a slanting position. Heat these 2 tubes in a bath of boiling water or gently over a Bunsen flame until the medium turns brown. Pour 1 tube into a sterile Petri dish and allow the other to cool in a slanting position. This is known as chocolate agar, and is used for culturing Pfeiffer's bacillus (*Hemophilus influenzae*).

2. Place 2 blood agar plates in the locker for future use. Divide the other 4 in halves by means of a glass-

pencil line drawn on the bottom of each plate. Inoculate each half-plate with one of the following organisms, according to the method demonstrated by the instructor: *Staphylococcus aureus*, *Staphylococcus albus*, *Streptococcus viridans*, *Streptococcus hemolyticus*, *Diplococcus pneumoniae* (pneumococcus), *Neisseria meningitidis* (meningococcus), *Neisseria catarrhalis*, and *Bacterium pneumoniae* (Friedländer's bacillus). Inoculate the chocolate agar plate with *Hemophilus influenzae* (Pfeiffer's bacillus). Invert all plates and incubate.

3. Discussion of respiratory organisms or demonstration.

EXERCISE 2

1. Describe colonies on blood agar and chocolate agar plates. Note particularly any changes in the blood agar, and compare the colonies on the several plates.

2. Prepare Gram stains of all organisms. Make smears from colony growths.

3. Inoculate tubes of milk, gelatin, and dextrose agar or dextrose broth with each organism.

4. Transfer all organisms to blood agar slants. Use the chocolate agar for Pfeiffer's bacillus.

EXERCISE 3

1. Descriptions of blood agar slants and media inoculated in Exercise 2, items 3 and 4.

2. Inoculate tubes of inulin broth with *Streptococcus viridans* and *Diplococcus pneumoniae*.

3. Bile-solubility test.—Use either fresh bile or bile which has been stored in the refrigerator for several weeks. If the latter is available, remove the clear liquid with a sterile pipette, avoiding both scum and sediment. Prepare suspensions of pneumococci and *Streptococcus viridans* by adding 5 cc. of salt solution (0.8 per cent) to the blood agar slant culture and carefully scrape off the growth with

a sterile loop needle. To 0.5 cc. of each suspension in an agglutination tube add 0.2 cc. of bile. Place in a water bath at 37° C. for 20 minutes and note whether or not the cells have been dissolved.

4. The salt suspension may also be used for agglutination tests when a type pneumococcus has been used. Pneumococcus typing may preferably be the subject of a demonstration. In this case the mice should be inoculated at this exercise and the demonstration completed the following day (see Sec. XVI).

EXERCISE 4

1. Make tonsillar and nasopharyngeal cultures from some member of the class, using sterile swabs supplied by instructor. If class work is done in pairs, use your partner. The instructor will demonstrate the method of taking the cultures.

2. Inoculate the blood agar plates prepared in Exercise 1 with the swabs, using the method shown by the instructor. It is preferable but not absolutely essential to prepare fresh blood agar plates for this experiment.

3. Completion of demonstration of mouse method of pneumococcus typing, including the examination by the class of smears from the heart blood of each mouse. Prepare both Gram and capsule stains from these smears. The capsule stain is made as follows (Hiss method): Prepare smear on a slide, allow to dry in air, and fix by heat. Stain for a few seconds with a solution of fuchsin (5 cc. of saturated alcoholic solution diluted with 95 cc. of distilled water). While the staining solution is on the slide, hold the preparation over a flame until it begins to steam. Wash off the dye with a 20 per cent solution of copper sulphate in water. Blot dry. Do not wash in water. Better results are sometimes obtained by omitting the heat fixation.

4. Descriptions of cultures (see Exercise 3, items 1 and 2).

EXERCISE 5

1. Examine blood agar plates from Exercise 4, item 2. Note the types of colonies and relative proportion of each type.

2. Prepare Gram stains from at least 2 colonies of each type, and note morphology and Gram reaction.

3. Make bile solubility tests on at least 2 colonies of the "green-producing" type. The suspension for this test may be prepared by picking off a colony with a straight inoculating needle and emulsifying in 0.5 cc. of salt solution in an agglutination tube.

NOTE.—If time permits, transfers from these plates may be made to blood agar slants and the organisms further identified in subsequent exercises. It is also possible to introduce unknown cultures or mixture at this point. The first inoculations from these "unknowns" should be on blood agar plates.

SECTION VIII

MISCELLANEOUS AND HIGHER BACTERIA

FOUR TO NINE EXERCISES

EXERCISE 1

1. Inoculate agar slants with *Bacillus anthracis* and with some member of the hemorrhagic septicemia group.

2. Inoculate glycerin agar slants (nutrient agar containing 10 per cent glycerin) with *Mycobacterium tuberculosis* (the tubercle bacillus), *Mycobacterium leprae*, and *Mycobacterium moelleri.*

3. Inoculate Löffler's blood serum, or 2 per cent glycerin agar, with *Corynebacterium diphtheriae* (the diphtheria bacillus) and *Corynebacterium hoffmanii.*

4. Prepare and examine stains of a streptothrix, an actinomyces, a fusiform bacillus, and a blastomyces.

5. Demonstration of action of a diphtheria culture and of diphtheria toxin on guinea pigs, both unprotected and protected with diphtheria antitoxin. Injections should be made at this exercise and autopsies at subsequent exercise (see Sec. XV).

EXERCISE 2

1. Prepare Gram stains from cultures inoculated in Exercise 1, item 1. Retain culture of *Bacillus anthracis* for later exercise.

2. Prepare Löffler's methylene blue[1] and Neisser stains (or any modification of the Neisser stain) of organisms of the diphtheria group (Exercise 1, item 3). The Neisser stain is prepared as follows:

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[1] Methylene blue solution 10,000 parts, NaOH 1 part.

METHYLENE BLUE SOLUTION

Methylene blue	0.1	gm.
Alcohol (95 per cent)	2	cc.
Glacial acetic acid	5	cc.
Distilled water	95	cc.

Dissolve the methylene blue in the alcohol and add this solution to the acetic acid–water mixture.

BISMARCK BROWN SOLUTION

Bismarck brown	0.2	gm.
Water	100	cc.

Dissolve the dye in boiling water and filter.

Fix the preparation on a slide. Stain with the methylene blue solution for 1 minute. Wash in water and counterstain with the Bismarck brown solution for 30 seconds. Wash, dry, and examine.

3. If Section II was omitted, prepare India-ink stain of tartar collected from the teeth (see Sec. II, Exercise 2).

4. **Examine gonorrheal smears.**—Use the Gram stain and report as positive or negative, for gonococci.

5. Autopsies of guinea pigs injected in Exercise 1, item 5.

6. Demonstration of the dark-field microscope.

EXERCISE 3

1. **Examination of unknown diphtheria cultures.**—These may usually be obtained from the local or state Department of Health laboratories. Report results as positive or negative for diphtheria-like bacilli.

EXERCISE 4

1. **Prepare stains from cultures of members of the acid resisting group** (Exercise 1, item 2). The stain is made as follows: Prepare smear in the usual manner. Flood with carbolfuchsin and heat over a low flame or a steaming

water bath for 5 minutes. Replace stain as evaporation occurs. Do not allow slide to become dry. Wash off stain and cover the stained preparation with acid alcohol (2 per cent sulphuric acid in 95 per cent alcohol) until decoloration is almost complete. Wash, dry, and examine.

2. Examine several samples of sputum from tuberculosis patients. Use the stain described in item 1, except that after the final washing the preparation should be counterstained with Löffler's methylene blue. Report results positive or negative for the tubercle bacillus.

3. Spore stain of *Bacillus anthracis* culture (see Sec. III, Exercise 6).

4. **Demonstration.**—Autopsy of tuberculous guinea pig.

EXERCISES 5 AND 6

If the time available for laboratory work is too short to allow the student to carry through a Wassermann test, it is desirable to spend two exercises in demonstrating and discussing this technic (see Sec. XIII). Other tests for syphilis may also be shown, as, for example, the Kahn test and the Lange colloidal gold reaction (see Sec. XIII).

EXERCISES 7–9

Because of the great importance of diseases such as malaria, hookworm, and amoebic dysentery, it is essential that the medical student be given opportunity to examine preparations illustrating these diseases. If fresh material is not available, slides can be purchased through biologic supply-houses. One to three exercises can be spent profitably in this work.

SECTION IX

ANAEROBES

THREE EXERCISES

EXERCISE 1

1. Inoculate the following media from cultures of *Clostridium welchii*, *Clostridium sporogenes*, *Clostridium tetani*, and *Clostridium botulinum*. Use deep brain or beef heart medium, deep agar (shake culture), dextrose broth with fermentation vials, or dextrose agar (stab or shake culture) and milk. Before inoculation the media should be heated for 10 minutes in a bath of boiling water and allowed to cool without shaking. The medium in the inoculated tubes may be covered with a thin layer of sterile vaseline or mineral oil. For a general course it is desirable to omit *Clostridium tetani* and *Clostridium botulinum* since these organisms produce powerful toxins.

2. Other methods of cultivating anaerobes should be demonstrated or discussed.

3. **Demonstration of intravenous injection of a rabbit with a culture of *Clostridium welchii*.**—The animal should be killed a few minutes after injection and the body placed in an incubator.

4. **Demonstration of the presence of botulinum toxin in a canned food and of the method of typing the toxin.**—Canned spinach or string beans may be inoculated with a strain of *Clostridium botulinum* at a previous exercise as a demonstration, or the material may be prepared by the instructor. Either guinea pigs or white mice may be used. (see Sec. XV). If material from canned foods is not avail-

able, a beef heart or liver broth culture may be used. The demonstration is completed in the next exercise.

EXERCISE 2

1. **Autopsy of rabbit injected with *Clostridium welchii*.**—The student should make smears from the liver, stain with carbolfuchsin, and examine.

2. Completion of demonstration of the presence of botulinum toxin in a canned food or culture and of typing the toxin.

3. Discussion of results of demonstrations.

EXERCISE 3

1. Descriptions of cultures inoculated in Exercise 1, item 1.

2. Prepare Gram stains and hanging drops of cultures inoculated in Exercise 1, item 1.

3. Prepare spore stains from cultures of *Clostridium welchii* and *Clostridium sporogenes* (see Sec. III, Exercise 6).

4. Demonstration of the action of tetanus toxin (see Sec. XV).

SECTION X

EXAMINATION OF WATER AND MILK

FIVE EXERCISES

EXERCISE 1

1. **Water examination.**—Obtain samples of water from instructor. It is desirable to have available one sample of good water and one of polluted water. Prepare dilutions of the samples in sterile tap water or sterile 0.8 per cent salt solution. Make 1–10 and 1–100 dilutions of each sample.

2. **Colony count.**—Plate 1 cc. of original sample and 1 cc. of each dilution, using 4 plates for each sample or dilution. To plate, place 1 cc. portion in a sterile Petri dish and pour into the dish about 10 cc. of agar which has been melted in flasks or tubes and cooled to 45° C. Mix contents of plates by gently tipping from side to side before the agar has solidified. Allow to harden, invert, and place 2 plates from each 1 cc. portion in the incubator and 2 at 20° C. or in the laboratory locker.

3. **Determination of *Bacterium coli.***—To each of 5 tubes containing an inverted vial and not less than 10 cc. of lactose broth, add 10 cc. of original sample of one water. Use sterile pipettes. It is convenient to use potato tubes for large amounts of lactose broth but any large culture tube will serve as well. Repeat with 1 cc. portions of water and 1 cc. portions of the 1–10 dilution. Ordinary culture tubes containing lactose broth and an inverted vial may be used for these portions. Repeat the foregoing with the other sample of water. Incubate all tubes at 37° C.

EXERCISE 2

1. Count the colonies on the 37° C. plates (Exercise 1, item 2), and record the number of bacteria per cc. of water.

2. Record gas formation in all lactose broth tubes (Exercise 1, item 3). Replace tubes in incubator.

3. Prepare 4 Endo plates (see Sec. V, Exercise 5, item 2).—Eosin methylene blue medium may be substituted for Endo.

4. Inoculate Endo plates from lactose broth tubes showing gas in the vials. From 4 to 8 tubes should be used. It is not necessary to use a whole Endo plate for each tube, provided the plates are properly divided by glass-pencil lines and labeled.

EXERCISE 3

1. Count water plates held at 20° C. (Exercise 1, item 2).

2. Record gas formation in lactose broth tubes. For class work it is not necessary to confirm *Bacterium coli* in all tubes showing gas. This option is left to the instructor.

3. Examine Endo plates and record number showing typical *Bacterium coli* colonies.

4. Transfer 1 typical colony from each plate to lactose broth and to an agar slant.

5. **Milk examination.**—Plate on plain agar in duplicate 1 cc. each of dilutions of samples of milk, according to directions of instructor. The dilutions required will depend upon the source of the sample of milk. For ordinary raw milks, dilutions of 1–1,000, 1–10,000, and 1–100,000 are suggested. For certified milk, dilutions of 1–10 and 1–100 are usually sufficient. For pasteurized milks, dilutions of 1–100, 1–1,000, and 1–10,000 will be found convenient. Incubate the plates at 37° C. for 48 hours.

EXERCISE 4

1. Note gas formation in lactose broth tube from Exercise 3, item 4.

2. Prepare Gram stains from agar slant cultures (Exercise 3, item 4). Note not only the Gram reaction but also the presence or absence of spores.

3. Record confirmation of *Bacterium coli* on the basis of items 1 and 2.

4. **Differentiation of fecal and non-fecal strains of *Bacterium coli*.**—Make transfers from agar slants (item 2) to peptone and sodium citrate media. The peptone medium is prepared as follows: Dissolve 5 gm. of Difco peptone, 5 gm. of dextrose, and 5 gm. of dipotassium hydrogen phosphate (K_2HPO_4) in 800 cc. of distilled water. Heat gently for 20 minutes, filter through paper, cool, and dilute to 1,000 cc. with distilled water. Tube in 10 cc. portions and sterilize in the Arnold on three successive days.

To prepare the sodium citrate medium, dissolve 1.5 gm. sodium ammonium phosphate (microcosmic salt), 1 gm. potassium dihydrogen phosphate (KH_2PO_4), 0.2 gm. magnesium sulphate, and 3 gm. crystallized sodium citrate in 1 liter of distilled water. Tube and sterilize.

5. Inoculate tubes of milk with *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, and *Streptococcus lacticus*. Incubate.

EXERCISE 5

1. Count colonies on milk plates and report the number of bacteria per cc. of milk.

2. Stain smears made from milk cultures in Exercise 4, item 5, with carbolfuchsin or Löffler's methylene blue. Examine and describe the morphology.

3. **Methyl red test.**—It is desirable to incubate cultures for this test and the two following tests for three or four days, since 24-hour tests may be misleading. If time permits, items 3, 4, and 5 should be held over for a subsequent exercise. The tests are given here since they are concerned with water analysis.

Divide the peptone broth culture into two portions. To one portion add 5 drops of methyl red indicator (0.02 per cent alcoholic solution). Note reaction. Fecal coli usually give an orange-red color due to acid formation. Save the other portion of the culture for item 4.

4. **Voges-Proskauer reaction.**—To the other portion of the culture from item 3 add 5 cc. of 10 per cent potassium hydroxide solution and shake. Fecal strains of coli usually fail to give a reaction. Non-fecal strains give a deep-pink color.

5. **Sodium citrate test.**—Note presence or absence of growth in the sodium citrate medium. Coli strains of fecal origin usually fail to grow. Non-fecal strains generally grow well.

6. Report confirmation of fecal strains of coli isolated from water sample on the basis of items 3, 4, and 5.

SECTION XI[1]

VACCINE PREPARATION; AGGLUTINATION AND PRECIPITATION TESTS

TEN EXERCISES

EXERCISE 1

1. **Preparation of a vaccine.**—Make a suspension of *Bacterium coli* by adding 5 cc. of sterile salt solution (0.75–0.85 per cent) to an agar slant culture. Wash off the growth with the salt solution by rotating the tube vigorously while it is held in a vertical position. Allow clumps to settle. With a sterile pipette add 2 cc. of the bacterial suspension to 5 cc. of salt solution in a sterile tube. The suspension should be distinctly cloudy but not so heavy that there is appreciable spontaneous sedimentation of the bacteria. An inoculating needle held between the suspension and an incandescent electric lamp within an inch or two of the suspension should be just visible. Heat the suspension in a water bath at 60° C. for 45 minutes. Count the bacteria by one of several methods. Their number may be determined by direct microscopic counting, using a hemocytometer cell; by measuring the turbidity of the suspension against suspensions of bacteria or of barium chloride or sulphate which have been previously standardized; or by the following simple method: Draw into a capillary pipette equal volumes of blood, sodium citrate solu-

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[1] In the experiments for this and subsequent sections the student will be supplied with normal and "immune" reagents which he cannot prepare in the time available in the course. The reagents should be accompanied by memoranda upon the methods used in their preparation. These should be entered in the student's notebook along with records of the results of the experiments.

tion (1 per cent in 0.8 per cent sodium chloride solution), and heated bacterial suspension. (This is easily accomplished by drawing one of the reagents into the capillary up to a mark about 3 cm. from the tip, admitting an air bubble and drawing the next reagent to the mark. A drop of blood from a washed finger or ear pricked with a sterile point will suffice.) Mix the reagents in the pipette or on a slide. Prepare a smear from this material and allow it to dry in air without heating. Cover for 1 minute with Jenner's stain,[1] add an equal volume of distilled water, and allow to stay at room temperature for 5 minutes. Wash in distilled water for 1 minute and dry. Place under a microscope and count the number of bacteria and the number of red blood cells in at least 25 fields until at least 200 red blood cells have been counted. Assuming the blood contains approximately 5,000,000 red cells per c.mm. (5,000,000,000 per cc.), calculate the number of bacteria per cc. of suspension. Dilute the vaccine with sterile salt solution so that it will contain 2,000,000,000 bacteria per cc. Add a preservative (phenol or tricresol to give a final concentration of 0.2 to 0.5 per cent). Test the vaccine for sterility by inoculating an agar slant and an agar shake culture each with a loop of material. Incubate the slant and shake cultures at 37° C.

EXERCISE 2

1. Finish preparation of vaccine. Report results of counts and of sterility tests to instructor. Seal the vaccine in sterile ampules. Label adequately and preserve in the ice chest.

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[1] Jenner's stain, Leishman's modification. This is a modified Wright's stain. Dissolve 1 gm. of methylene blue in 100 cc. of distilled water, and 0.5 gm. sodium carbonate, and heat the solution at 65° C. for 12 hours. Then allow to stand for ten days at room temperature. To this add an equal volume of eosin solution (0.1 per cent eosin, yellow, in water), mix, and allow to stand for 6–10 hours with frequent stirring. Collect the precipitate on a filter and dry. Prepare the staining solution by dissolving 0.15 gm. in 100 cc. of methyl alcohol.

2. Demonstrations by instructor, such as injections into laboratory animals—intracutaneous, subcutaneous, intraperitoneal, intravenous, intracardial, and intraspinal. Bleedings from superficial veins and from the heart.

EXERCISE 3

1. **Macroscopic agglutination test.**—(With partner.) *Warning: The bacteria used in these tests are alive. Take the necessary precautions to avoid drawing any of the suspensions into the mouth. Wash the hands with soap and water and with lysol solution whenever it is suspected or known that the hands have been contaminated.*

Label a series of serologic tubes—A, B, C, D, E, and F. Place in each tube 0.5 cc. of salt solution (0.8 per cent NaCl in water). To tube A add 0.5 cc. of diluted anti-serum from a burette. Mix the contents of tube A thoroughly by alternately drawing up the material into a pipette and carefully running it back into the tube. Transfer 0.5 cc. of this mixture to tube B and mix as before. Transfer 0.5 cc. of the mixture in tube B to tube C and repeat the procedure through tube E. Withdraw 0.5 cc. of the mixture in tube E and discard it. Do not add any serum to tube F; it is the control tube to determine whether there will be any agglutination without serum. There should be 0.5 cc. of liquid in each tube. To each tube add from a burette 0.5 cc. of a suspension of bacteria in 0.8 per cent NaCl solution. Stir the mixtures in the tubes by carefully shaking the tubes until the mixtures are homogeneous. Incubate the tubes for 1 hour in a water bath at 55° C. Examine the tubes and record the presence or absence of agglutination. Hold the tubes in the ice chest over night and re-examine for agglutination.

NOTE.—The instructor may provide for this experiment any agglutinating serum of known titre and its homologous bacterial antigen.

2. Microscopic agglutination.—After the tubes have been incubated 15 minutes (in item 1 above), withdraw a loop of material from each; prepare hanging drops and examine under the microscope. Record motility and agglutination.

3. Microscopic agglutination (Widal method).—Mix on cover slips a loop of anti-typhoid serum (diluted 1:25 or 1:50 with salt solution) and a loop of normal serum each with a loop of suspension of *Bacterium typhosum.* Mount on hanging-drop slides. Allow to stand at room temperature for 1 hour. Examine under the microscope for agglutination.

NOTE.—When the test is conducted with typhoid bacilli and serum from the blood of a patient suspected of having typhoid fever, it is called the "Widal test."

Other bacteria and the corresponding anti-sera may be substituted for *Bacterium typhosum* and anti-typhoid serum.

EXERCISE 4

1. Group agglutinins.—Prepare suspensions of *Bacterium coli, Bacterium typhosum, Bacterium paratyphosum A, Bacterium paratyphosum B,* and *Proteus vulgaris.* Prepare dilutions of the following sera by the method given in Exercise 3, item 1: anti-coli, anti-typhoid, and anti-paratyphoid A and B. Determine the maximum dilution of each serum which suffices to give complete, nearly complete, and slight agglutination of each of the bacterial species. Tabulate the results and present them to the instructor with a brief discussion of group agglutinins. If more than six dilutions (A–F) of each serum are used, the student is more likely to observe the so-called "zone phenomenon." It may be missed when only a few widely spaced dilutions are used.

NOTE.—Pathogenic bacteria used in these and similar experiments may be first killed with formaldehyde. Add 0.2 cc. of a 40 per cent solution to each 10 cc. of bacterial suspension.

EXERCISE 5

1. **Effect of salt on agglutination.**—Prepare 15 cc. of a suspension of *Bacterium typhosum* in 0.8 per cent NaCl solution as in Exercise 4. (*a*) Save 5 cc. of the suspension. (*b*) To 5 cc. add 5 cc. of distilled water. (*c*) To another 5-cc. portion add 5 cc. of an agglutinating dilution of an antityphoid serum. (*d*) Sediment the bacteria in (*b*) and (*c*) by centrifugation, resuspend them in distilled water, and repeat twice again. If clumps are present after the last resuspension of the bacteria, alternately suck up and blow out the material through a small-tipped pipette until the suspension is homogeneous. Arrange the reagents shown in Table III in 15 serologic tubes. Incubate the tubes in the water bath at 45° C. for 1 hour. Examine for agglutination; record results and write a brief discussion of their significance.

TABLE III

Three Sets of Five Tubes	First Set; Add to Each Tube	Second Set; Add to Each Tube	Third Set; Add to Each Tube
1. 0.5 cc., distilled water 2. 0.5 cc., diluted agglutinating serum 3. 0.5 cc., 0.8 per cent NaCl 4. 0.5 cc., 0.5 per cent $ZnSO_4$ 5. 0.5 cc., 10.0 per cent $ZnSO_4$	0.5 cc. of the original bacterial suspension (*a*)	0.5 cc. of the washed suspension (*b*)	0.5 cc. of the washed suspension (*c*)

2. **Effect of heat on serum agglutination.**—Place 0.5 cc. quantities of the reagents shown in Table IV in a series of serologic tubes. To each tube add 0.5 cc. of a suspension

of *Bacterium typhosum* (live or killed, as in Exercise 4) in salt solution. Incubate the tubes at 45° C. for 1 hour. Examine for agglutination; record results and write a brief discussion of the experiment and the results.

TABLE IV

Tube	
1	Undiluted anti-typhoid serum
2	Same as 1, heated at 56° C. for ½ hr.
3	Same as 1, heated at 65° C. for ½ hr.
4	Same as 1, heated at 73° C. for ½ hr.
5	Anti-typhoid serum diluted 1:10 with salt solution
6	Same as 5, heated at 56° C. for ½ hr.
7	Same as 5, heated at 65° C. for ½ hr.
8	Same as 5, heated at 73° C. for ½ hr.

EXERCISE 6

1. Removal of agglutinating power of a serum with bacteria ("agglutinin absorption").—The student should have prepared or should be provided with a serum which displays agglutinating power for two or more types of bacteria. This may be made available by preparing serum from an animal which has been "immunized" against two or more antigens or by mixing two or more specific agglutinating sera. The specific agglutinating titre of the polyvalent serum for each type of organism (i.e., *Bacterium typhosum* and *Bacterium coli*) should have been determined by the method given in Exercise 3 before beginning this exercise.

Prepare 4 sets of serologic tubes, 6 in each set. Arrange them in 4 rows in a rack. In the first 5 tubes in each of the rows arrange a series of 5 dilutions of the serum—1:20, 1:40, 1:80, 1:160, and 1:320. The sixth tube in each row should receive 0.8 per cent NaCl solution and serve as a control. Each tube should contain 1.5 cc. of fluid (diluted serum or salt solution).

To each of the 6 tubes in rows 1 and 2 add 0.1 cc. of a very heavy suspension of *Bacterium typhosum;* and to each of the tubes in rows 3 and 4 add 0.1 cc. of a very heavy suspension of *Bacterium coli.* Hold all the tubes in the water bath at 37°–45° C. for 2 to 4 hours and place in the ice chest. (To be completed at the next exercise.)

EXERCISE 7

1. **Completion of "agglutinin absorption."**—Place all the tubes in a centrifuge and whirl until the bacteria have been thrown down. Decant the supernatant fluid in each tube to a similarly labeled one in a series of clean tubes arranged as was the first set in Exercise 6. To each tube in rows 1 and 3 add 0.1 cc. of a moderately thick suspension of *Bacterium typhosum* in NaCl solution; to each tube in rows 2 and 4 add 0.1 cc. of a similar suspension of *Bacterium coli.* Hold all the tubes 1 hour in a water bath at 37°–45° C. Examine them for agglutination and record the results. Hold them in the ice chest over night, re-examine them, and record results. Write a brief discussion of this experiment.

2. The experiment on the absorption or removal of agglutinating power from a serum may be conducted more precisely and the dynamics of the reaction may be more explicitly investigated if more time is available to the student. The following procedure may be followed: (*a*) Determine the agglutinating titre of the serum for each of a series of antigens. (*b*) Prepare a standardized (counted) suspension of bacteria of each type being studied in the antigen series (see Exercise 4). (*c*) Determine the "absorption unit" for each bacterial suspension. The "absorption unit" may be taken as the minimal quantity of a standardized suspension which will just suffice to reduce the agglutinating titre of the serum for this suspension by 90 per

cent (or by 95 per cent, at the instructor's discretion). (*d*) Determine the effect of 1, 2, 5, 10, etc., absorption units of each suspension upon the agglutinating titres of the serum for each bacterial type being studied.

3. If any time remains in this exercise, the student should be given an opportunity to perform animal injections. If the student is to perform later some of the complement fixation or anaphylaxis experiments, he should begin the immunization or sensitization of animals against one or more antigens. The student should continue this on a program which has been approved by the instructor.

EXERCISE 8

1. **Titration of a precipitating serum (flocculation method).**—Arrange in a rack a series of 11 serologic tubes. To each of 10 tubes add 0.1 cc. of a serum prepared from an animal which has been "immunized" or has been "sensitized" against egg albumin; to the eleventh tube add no serum (see Sec. XVII). There should be available or should be prepared by the student the following solutions of albumin: (1) 3 per cent, (2) 0.3 per cent, (3) 0.03 per cent. To tubes 1, 2, and 3 add 0.5, 0.25, and 0.1 cc. quantities, respectively, of albumin solution (1); to tubes 4, 5, and 6 add similar quantities of solution (2); and to tubes 7, 8, and 9 add similar quantities of solution (3). To tube 10 add 0.5 cc. of 0.8 per cent NaCl solution. To tube 11 add 0.5 cc. of albumin solution (1). (Tube 11 contains no serum and serves as a control upon the flocculating effect of NaCl solution upon egg albumin.) To each tube add the quantity of NaCl solution necessary to bring the total volume to 1.0 cc. Hold all the tubes in a water bath at 37°–45° C. for 1 hour. Examine them for flocculi, record results, and place the tubes in the ice chest. Re-examine the tubes next day and record the results. Calculate the

final dilution of the albumin in each tube. Arrange a tabular outline of the experiment. Write a brief discussion of the results.

2. **Titration of a precipitating serum (ring test).**—The instructor should provide an anti-albumin precipitating serum whose ring-test titre has been determined. With a capillary pipette introduce into each of 5 "precipitin" or capillary test tubes 0.2 cc. of the serum. Into a sixth tube introduce 0.2 cc. of 0.8 per cent NaCl solution. With another capillary pipette add to the first 5 tubes 0.2 cc. quantities of albumin solutions of concentrations so chosen by the instructor that positive and negative results will be obtained by the student with the serum which is provided. The sixth tube, containing salt solution in place of serum, should receive 0.2 cc. of the most concentrated albumin solution used in the experiment. The albumin solutions should be run into the tubes carefully so that there is a distinct ring of contact between the albumin solution and the serum in each tube. Examine the tubes for a cloudy ring 5–10 minutes after the solutions are added, after incubation for 1 hour in a water bath at 37°–45° C., and after being held in the ice chest over night. Throughout the course of the experiment no turbidity should become apparent in the sixth (control) tube, which contains no serum. If a slight zone of opacity develops at the interphase between salt and albumin solutions in the control tube, special care should be exercised in interpreting apparently positive reactions in the other tubes.

EXERCISE 9

NOTE.—Each student should perform the experiments in item 3 and in either item 1 or item 2.

1. **Effect of heating upon the precipitating power of a serum.**—Arrange and perform an experiment with a pre-

cipitating serum and its homologous antigen similar to the experiment with an agglutinating serum (Exercise 5, item 2).

2. **Effect of salt upon the precipitating power of a serum.**—Arrange and perform an experiment similar to Exercise 5, item 1, following the directions of the instructor.

3. **Forensic precipitation test.**—This is merely a special application in medico-legal work of the specific precipitation technique to the identification of blood stains, meat adulteration, etc. In conducting the test it is necessary that special precautions be taken. It is desirable that there should be available sera which produce precipitation of the homologous antigen within 15–20 minutes even when the latter has been diluted 1,000 or 10,000 times with salt solution. For the experiment the student should be provided with serum from an animal which has been immunized against human blood and at least one anti-serum against another blood. The antigens to be identified may be provided in the form of dried-blood stains on paper or cloth or as bits of meat. The procedure to be followed may be illustrated by the case when the student has been supplied with two rabbit sera which precipitate, respectively, with human and with sheep-blood protein, and with two blood-stained cloths—one with human blood and another with the blood of some laboratory animal. The student should identify the human blood stain and should determine whether the second blood stain was prepared with sheep or with some other blood.

The blood should be extracted from each cloth by being shaken in a clean test tube with sodium chloride solution until—after vigorous shaking—there is a persistent or permanent foam. (This indicates that the proteins of the blood stain are probably present in concentration of at least 0.1 per cent.) The salt-solution extract may then be used as antigen in precipitation tests with each of the sera, using the ring-test method described in Exercise 8, item 2.

EXERCISE 10

1. **Macroscopic hemagglutination.**—This is a simple test for compatibility of the bloods of donor and recipient before a transfusion. The experiment may be performed by the student with the bloods of laboratory animals or (at the discretion of the instructor) with samples of blood taken from various members of the class by pricking a finger.

TABLE V*

Tube	Serum A	Serum B	Cells A	Cells B	0.8 Per cent NaCl Solution
1	5			1	20
2		5	1		20
3	5		1		20
4		5		1	20
Controls					
5			1		25
6				1	25

* Quantities are given in terms of drops from pipettes which deliver approximately 20 drops per cc.

Each sample of blood should be collected in part in citrate solution to provide red blood cells, and in part in a clean, dry test tube to provide serum. The quantities to be collected must be determined by the number of students who will test each sample. To obtain cells: Collect approximately 1 cc. in 2 cc. of sodium citrate solution (1 per cent in 0.8 per cent NaCl solution). To obtain serum: Collect 1 cc. in a dry, clean test tube, permit the blood to clot, and remove the serum to another clean tube with a clean capillary pipette. The red blood cells in the citrate solution should be sedimented by centrifugation, the supernatant liquid removed, salt solution added, and the separation repeated once again. The sedimented cells should then be resuspended in enough salt solution to make up a total volume of 1 cc. To perform the tests for hemagglutinating properties of the several sera against the heterologous and homologous cells, set up the mixtures shown in Table V in serologic tubes (A represents the donor and B the recipient in an hypothetical transfusion).

Place all the tubes in a water bath or incubator. Examine for agglutination of the red cells each half-hour for 1–3 hours. Record the findings of each examination.

TABLE VI*

Cover Glass	Serum Type II	Serum Type III	Red Blood Cell Suspension	0.8 Per Cent NaCl Solution
1.	....	....	1	2
2.	2	....	1	....
3.	....	2	1	....

* Quantities are given in terms of loops.

2. **Microscopic blood grouping.**—(This experiment may be performed while the tubes of the first experiment of this exercise are being incubated.) The instructor should provide sera known to be of Types II and III, respectively. Suspensions of red blood cells from members of the class should be prepared by the method given in item 1 of this exercise. (If suspensions have been prepared for item 1, above, they may be used in this experiment.) Three clean cover glasses and hollow-ground slides should be prepared. Arrange the mixtures shown in Table VI on the cover glasses. Mix the reagents on the cover glasses with toothpicks, wires, or loops, *being careful not to carry material over from one glass to the next.* Invert a vaselined slide over each glass as in preparing a hanging drop (see Sec. II,

Exercise 1). Examine the hanging drops under the microscope (with "high-dry"—2/3—objective) after they have been at room temperature for 15 minutes. In determining whether or not agglutination of the erythrocytes has occurred, care should be exercised against confusing *rouleau* formation with agglutination. Record the results and determine from them the blood grouping of the tested cells in Jansky and in the Moss classifications.

SECTION XII

HEMOLYSIS; BACTERIOLYSIS; COMPLEMENT FIXATION

TWELVE EXERCISES

EXERCISE 1

1. **Test of fragility of erythrocytes.**—Each student should conduct this experiment, using red cells from at least two species of animals.

Prepare a series of NaCl solutions of graded concentrations from a stock 1 per cent solution. Thus, to 9.5, 9.0, 8.0, 7.8, 7.6, 7.4, 7.2 . . . . 5.0 cc. of the stock solution add 0.5, 1.0, 2.0, 2.2, 2.4, 2.6, 2.8 . . . . 5.0 cc. of distilled water. (These solutions may be prepared and supplied by the instructor.) The student should calculate the final concentration of each salt solution.

For each suspension of erythrocytes to be tested: Arrange a series of clean, dry tubes in a rack, add 2.0 cc. quantities of the graded series of salt solutions to the tubes in a sequential array, add 0.05 cc. of the washed, sedimented cells (free of excess salt solution) to each tube, and mix thoroughly. Hold the tubes in the ice chest over night. Examine them for hemolysis and record the results.

2. If time remains in Exercise 1, it may be spent profitably in a review discussion of the structure, function, etc., of erythrocytes, the theories of the mechanism of their tonicity and of plasmolysis, plasmoptysis, etc.

EXERCISE 2

1. **Simple hemolysis experiment.**—Prepare a series of serologic tubes, A–G, as for an agglutination experiment,

i.e., 0.5 cc. of 0.8 per cent NaCl solution in each tube; add 0.5 cc. of a prepared hemolytic serum to tube A, mix, withdraw 0.5 cc., and transfer to tube B; repeat the process through tube E; discard 0.5 cc. from tube E so that tubes F and G (control tubes) receive no serum. To each of the tubes A–F, inclusive, add 0.5 cc. "complement" (fresh, diluted, guinea-pig serum). Then to each tube, A–G inclusive, add 0.5 cc. of a $2\frac{1}{2}$ per cent suspension of red blood cells in 0.8 per cent NaCl solution. Mix by carefully shaking the tubes. Incubate them 1 hour at 37°–45° C. in a water bath. Be sure to add the reagents in the order specified. Prepare a diagrammatic outline of the experiment. (Observe that tube F is a control to show that "complement" does not of itself cause hemolysis; tube G is a control to show that NaCl solution of itself is not hemolytic.) After the incubation period, examine the tubes for hemolysis and record the results of the examination in the following terms: −, no hemolysis; +, a trace of hemolysis; ++, considerable hemolysis; +++, nearly complete hemolysis; ++++, complete hemolysis.

EXERCISE 3

1. **Hemolytic action of normal serum.**—The student or instructor should provide for this experiment 1 cc. fresh dog serum, 5 cc. of a 2 per cent suspension of washed guinea-pig erythrocytes, and 5 cc. of a 2 per cent suspension of washed human erythrocytes.

Place 0.3 cc. of dog serum in each of 2 serologic tubes. Inactivate the serum in 1 tube by holding it in a water bath at 56° C. for 30 minutes. Add enough 0.8 per cent NaCl solution to the serum in each of the 2 tubes to make the final volume in each 3.0 cc. The serum is now diluted 1:10. Arrange in a series of 8 serologic tubes the reagents shown in Table VII. Incubate all 8 tubes at 37°–45° C. for

1 hour. Examine the tubes for hemolysis after 5, 20, 25, 30, and 60 minutes' incubation. Record the results and write a brief discussion of the experiment.

NOTE.—This experiment may be amplified by the instructor by including the study of several sera, red cells from other animals, and by using more than one dilution of serum.

TABLE VII*

TUBE	DOG SERUM		GUINEA-PIG CELLS	HUMAN CELLS	0.8 PER CENT NaCl SOLUTION
	Active	Inactive			
1	0.5	....	0.5	....	....
2	.5	....	....	0.5	....
3	....	0.5	.5	....	....
4	....	.5	....	.5	....
5	.05	.5	....	.5	....
6	0.05	0.5	.5	....	....
7	....	....	0.5	....	0.5
8	....	....	....	0.5	0.5

* Quantities are in cubic centimeters.

EXERCISE 4

1. Bactericidal action of normal and of immune serum.—It is suggested that the class be divided into three sections and that students in each conduct the experiment with one of the following sera: (1) fresh, normal rabbit serum; (2) rabbit anti-typhoid serum; and (3) rabbit anti-staphylococcus serum. The animals from which the two immune sera are obtained should have been immunized by (as nearly as possible) the same technique. The student should have available for this experiment 24-hour cultures of *Bacterium typhosum* and of *Staphylococcus aureus* and about 4 cc. of serum.

Place 0.5 cc. of serum in each of 4 serologic tubes. Inactivate the serum in 2 tubes by heating in a water bath at 56° C. for 30 minutes. While the serum is being inactivated, wash the bacterial growths off the agar slants with sterile salt solution and dilute the suspensions 1:1,000 with sterile salt solution. Arrange the reagents shown in Table VIII in 6 sterile tubes (culture tube size). Incubate all 6 tubes at 37° C. for 1–3 hours. While the tubes are incubating, melt some tubes of plain or glucose agar and cool them in a water bath at 42° C. At the end of the incubation period add the contents of a tube of melted, cooled agar to each of the 6 tubes and then pour to sterile Petri dishes. After the plates have hardened and set, invert them and place them in an incubator. Count the colonies on each after 24 and 48 hours' incubation. Record the results and write a discussion of the experiment.

TABLE VIII*

Tube	Serum		*Bacterium typhosum* (1:1,000)	*Staphylococcus aureus* (1:1,000)	0.8 Per Cent NaCl
	Fresh	Inactivated			
1.........	0.5	....	0.5	....	....
2.........	....	0.5	.5	....	....
3.........	....	....	0.5	....	0.5
4.........	0.5	....	....	0.5	....
5.........	....	0.5	....	.5	....
6.........	....	....	....	0.5	0.5

* Quantities are given in cubic centimeters.

Note.—Throughout this experiment an aseptic technique should be observed and only sterilized tubes, pipettes, plates, salt solution, etc., should be used.

The instructor may amplify the experiment by having the same or different students study the bactericidal actions of more than one dilution of each serum, and by including anti-sera against bacteria of other groups.

EXERCISE 5

1. **Specific (immune) hemolysis; titration of hemolytic serum (amboceptor).**—The amboceptor (Ehrlich) or sensitizer (Bordet) should have been inactivated by heat-

ing at 56° C. for 30 minutes. The complement (Ehrlich) or alexin (Bordet) should be a 1:20 dilution of fresh guinea pig serum, prepared from the pooled sera of at least three healthy guinea pigs. (The pigs should not have been fed for 12 hours before the bleeding.) The red blood cells should be sheep cells washed with 0.85 or 0.9 per cent NaCl solution and made up in salt solution to 2.5 per cent concentration by volume. All dilutions of reagents are made with the salt solution. It is not necessary to use precautions to maintain sterility in these tests. It is important that all glassware used shall have been thoroughly cleaned with cleaning solution, rinsed with tap water and distilled water and dried. Arrange the mixtures as indicated in Table IX.

TABLE IX*

DETERMINATION OF UNIT OF SENSITIZER OR AMBOCEPTOR

Tube	Inactivated Sensitizer (1:500)	Alexin (1:20)	Red Blood Cells (2.5 Per Cent)	0.85 Per Cent NaCl
1	0.05	1.0	1.0	To 3.0
2	.10	1.0	1.0	To 3.0
3	.15	1.0	1.0	To 3.0
4	.20	1.0	1.0	To 3.0
5	.25	1.0	1.0	To 3.0
6	.30	1.0	1.0	To 3.0
7	.35	1.0	1.0	To 3.0
8	.40	1.0	1.0	To 3.0
9	.45	1.0	1.0	To 3.0
10	.50	1.0	1.0	To 3.0
Controls				
11	0.50		1.0	To 3.0
12		1.0	1.0	To 3.0
13			1.0	To 3.0

* Quantities are given in cubic centimeters.

Incubate the tubes in a water bath at 37°–40° C. for 1 hour. Examine them for hemolysis and determine the unit of sensitizer. *The unit is taken as the smallest quantity of sensitizing serum that gives complete hemolysis.*

2. While the tubes are being incubated, the instructor may lead a discussion on the preparation of an immune or specific hemolytic serum.

EXERCISE 6

1. **Titration of alexin (complement).**—The complement used in Exercise 5 may be used here, provided these exercises come on the same or on succeeding days. The sensitizer or amboceptor titrated in Exercise 5 is diluted with sodium chloride solution so that the quantity equivalent to 1 unit is contained in 0.25 cc. In the complement titration (and, for complement fixation and Wassermann tests later, in antigen titrations) 2 units (0.5 cc.) are used. Arrange the mixtures as indicated in Table X.

TABLE X

DETERMINATION OF UNIT OF ALEXIN (COMPLEMENT)

Tube	Inactivated Sensitizer, in Units	Alexin (1:20)	Rbc. (2.5 Per Cent)	0.85 Per Cent NaCl
		cc.	cc.	cc.
1	2	0.10	1.0	To 3.0
2	2	.20	1.0	To 3.0
3	2	.30	1.0	To 3.0
4	2	.40	1.0	To 3.0
5	2	.50	1.0	To 3.0
6	2	.60	1.0	To 3.0
7	2	.70	1.0	To 3.0
8	2	.80	1.0	To 3.0
9	2	0.90	1.0	To 3.0
10	2	1.00	1.0	To 3.0
11	2	1.20	1.0	To 3.0
Controls				
12	2		1.0	To 3.0
13		1.0	1.0	To 3.0
14			1.0	To 3.0

Incubate the tubes in a water bath at 37°–40° C. for 1 hour. Examine them for hemolysis and determine the unit of alexin. *The unit is the smallest quantity of diluted* *alexin (complement) which causes complete hemolysis of 1 cc. of 2½ per cent suspension of the erythrocytes in the presence of two units of sensitizer.*

2. While the tubes are being incubated the instructor may demonstrate (or discuss) a series of experiments which portray the alleged tri-partite structure of complement and the function of "end-piece," "mid-piece," and "third piece."

EXERCISES 7, 8, 9

The student should arrange protocols for the following experiments and perform some of them, after approval by the instructor:

1. Absorption of sensitizer by erythrocytes; non-absorption of alexin by erythrocytes.
2. Absorption by erythrocytes of sensitizer *only* from a mixture of sensitizer and alexin.
3. Dependence of activity of alexin upon its concentration as well as upon the total amount present.
4. Dependence of sensitizer on the total amount present and not on its concentration.
5. The velocity of sensitizer absorption by erythrocytes by titrating for sensitizer separated from a sensitizer—red blood cell mixture after several time-intervals.
6. The liberation of sensitizer after the hemolysis of red blood cells by sensitized cells in the presence of complement.
7. The titration of alexin samples which have been held at several temperatures for various lengths of time.

EXERCISE 10

1. **Specific complement fixation.**—This test may be carried out with a protein antigen (egg albumin) and a homologous anti-serum (rabbit anti-ovalbumin serum);

or with a suspension of bacteria or with extractives of bacteria and specific anti-sera; or with one or the other of the so-called Wassermann antigens and normal human or syphilitic sera; etc.

In general, the complement fixation test is known as the "Bordet-Gengou phenomenon." When the complement fixation test is applied to the study of antibody-like property in the serum of a syphilitic, it is known as the Wassermann test and is a valuable adjunct in diagnosis. The principle of the test is the fixation, binding, or utilization of complement (alexin) in the presence of an antigen and its specific or homologous amboceptor (sensitizer).

If to such a system of reagents red blood cells and specific hemolytic sensitizer be added, no hemolysis results, apparently because there is no free or uncombined alexin for the hemolytic reaction. Hence, it is customary to refer to the hemolytic sensitizer and red cells as the indicator of complement fixation. If the various reagents which enter into the system are added only in necessary and sufficient amounts, complement fixation is said to be complete if there is no hemolysis, nearly complete if there is a trace of hemolysis, partial if there is considerable hemolysis, and negative if hemolysis is complete. When hemolysis is complete, the complement fixation is negative (−); when partial hemolysis occurs, the complement fixation is partially complete (++); when there is no hemolysis, complement fixation is complete (++++). Thus the extent of reaction in the indicator (hemolytic system) is a reciprocal measure of the extent to which alexin has been bound and, as the test is usually arranged, is a reciprocal measure of the presence of the specific sensitizer in the serum which is being examined.

The test is complicated by the necessity for "controlling" or eliminating the capacity of many antigens to bind alexin even in the absence of specific sensitizer and of many sensitizers to bind alexin in the absence of specific antigen.

2. **Non-specific binding of alexin by antigen.**—The instructor will supply an antigen and a specific anti-serum. To determine the non-specific alexin binding powers of the antigen, arrange in a series of serologic tubes various fractions of a cubic centimeter of antigen. To each add 2 units of alexin and enough 0.85 per cent NaCl solution to bring the volume of fluid in each to a minimal constant. Incubate the tubes in a water bath at 37°–40° C. for 1 hour. Add to each tube 1 cc. of a 2.5 per cent suspension of red blood cells and 2 units of specific hemolytic serum. Add NaCl solution to bring the total volume to 3 cc. Incubate the tubes in the water bath for 30 minutes. Examine them for hemolysis and record the results. The unit of antigen should be determined from the results after discussion with the instructor.

EXERCISE 11

1. **Determine the non-specific alexin binding power of the immune serum (sensitizer).**—Use the technique described in Exercise 10, item 2, substituting serum for antigen.

EXERCISE 12

1. **Specific complement fixation.**—Bordet-Gengou phenomenon. (The instructor should explain the nature of "anti-complementary" action.) The quantities of antigen and of anti-serum used in the test will be suggested by the instructor after an examination of the results of Exercises 10 and 11. The test may be conducted in two ways: (*a*) using various quantities of antigen and a constant quantity of anti-serum, (*b*) using a constant quantity of antigen and various quantities of anti-serum. It is advisable to have

half the students perform the test each way. A unit of alexin is used in each tube. After an incubation of 1 hour at 37°–40° C., to each tube is added 1.0+ cc. of a 2.5 per cent suspension of red blood cells which have been sensitized by the addition of inactivated hemolytic sensitizer in such quantity that 2 units of sensitizer are added to each 1.0 cc. of red cell suspension. (1.0+ cc. = 1.0 cc. of cells + 2 units of sensitizer.)

SECTION XIII

WASSERMANN REACTIONS; FLOCCULATION TESTS FOR SYPHILIS; COLLOIDAL GOLD TEST

SEVEN EXERCISES

EXERCISES 1–4

1. **Wassermann reactions.**—Before undertaking these experiments the student should have a clear comprehension of the nature of the antigens that are commonly used in the Wassermann test. It is not suggested that the antigen be prepared by the student in the time available in a course on serology. The procedure may be outlined by the instructor. Satisfactory antigens may be obtained from biologic supply houses. The instructor should provide sera known to be "Wassermann negative" and "Wassermann positive," respectively, in addition to sera whose reactions are to be determined by the student.

It is not essential—nor even suggested as a desirable procedure—that all students in a class conduct all of the titrations.

2. **Preliminary titrations of antigen.**—The students should determine: (*a*) the minimal quantity of antigen which is anti-complementary; (*b*) the minimal quantity of antigen which is hemolytic in the absence of alexin, and the largest quantity which is not hemolytic; (*c*, *d*) the antigen unit: the smallest quantity of antigen with which there is complete binding of 1.5 units of complement (i.e., no subsequent hemolysis after the doubly sensitized red cells are added) in the presence of 1 unit of serum known to be

(++++) syphilitic. If a titrated syphilitic serum is not available, approximately 0.1–0.2 cc. of serum known to be syphilitic may be used.

Titrations (*a*), (*b*), and (*c*) may be conducted along the lines indicated in Tables XI–XIV.

a) Arrange the mixtures as given in Table XI.

TABLE XI

TITRATION OF ANTI-COMPLEMENTARY ACTION OF ANTIGEN

Tube	Normal Human Serum	Antigen	Complement, in Units	Procedure	Rbc. 2½ Per Cent	Hemolytic Serum, in Units
	cc.	cc.			cc.	
1	0.1	0.5	1.5	Add NaCl solution to make volume in each tube 2.0 cc. Incubate at 37–40° C. for 30 minutes	1.0	2
2	.1	.6	1.5		1.0	2
3	.1	.7	1.5		1.0	2
4	.1	.8	1.5		1.0	2
5	.1	0.9	1.5		1.0	2
6	.1	1.0	1.5		1.0	2
7	.1	1.4	1.5		1.0	2
Controls	....	....	....		....	....
8	.1	....	....		1.0	2
9	.1	....	1.5		1.0	....
10	0.1	....	1.5		1.0	2
11	....	....	1.5		1.0	2
12	....	1.0	1.5		1.0	....
13	....	....	....		1.0	....

Then add NaCl solution to each tube to bring the total volume in each to 3.5 cc. (Table XI). Incubate at 37°–40° C. for 30 minutes. Examine for hemolysis and record results.

b) Arrange the mixtures as given in Table XII.

Then add NaCl solution to bring the volume in each tube to 3.0 cc. (Table XII). Incubate at 37°–40° C. for 30 minutes.

c, *d*) If a standardized antigen is available for reference, the syphilitic unit may first be determined as shown in Table XIII. Add NaCl solution to bring the volume in each tube to 3.0 cc. Incubate at 37°–40° C. for 30 minutes.

Examine for hemolysis and record results. The unit of syphilitic serum may be taken as the smallest quantity

TABLE XII

Titration of Hemolytic Action of Antigen

Tube	Antigen	Complement, in Units	Rbc. 2½ Per Cent
	cc.		cc.
1	0.5	1.5	1.0
2	0.8	1.5	1.0
3	1.0	1.5	1.0
4	1.5	1.5	1.0
5	2.0	1.5	1.0
6	2.5	1.5	1.0
Controls	....	....	....
7	....	1.5	1.0
8	....	....	1.0

TABLE XIII

Tube	Standard Antigen, in Units	Syphilitic (++++) Serum	Complement, in Units	Procedure	Rbc. 2½ Per Cent	Hemolytic Serum, in Units
		cc.			cc.	
1	1	0.2	1.0	Add NaCl solution to make volume in each tube 1.5 cc. Incubate at 37–40° C. for 30 minutes	1.0	2
2	1	.1	1.0		1.0	2
3	1	.05	1.0		1.0	2
4	1	.04	1.0		1.0	2
5	1	.03	1.0		1.0	2
6	1	.02	1.0		1.0	2
7	1	.01	1.0		1.0	2
8	1	.005	1.0		1.0	2
Controls	....	....	....		....	....
9	....	.05	1.5		1.0	....
10	....	.05	....		1.0	2
11	....	0.05	1.5		1.0	2
12	1	....	1.5		1.0	2
13	....	....	1.5		1.0	2
14	1	....	....		1.0	....
15	....	....	....		1.0	....

which in the presence of 1 unit of standard antigen fixes 1 unit of complement completely so that there is subsequently no hemolysis of the red blood cells in the presence of 2 units of specific hemolytic serum.

Using the now standardized syphilitic serum, the unit of a new antigen may be determined along the lines shown in Table XIV.

TABLE XIV

Tube	Syphilitic Serum, in Units	Antigen	Complement, in Units	Procedure	Rbc. 2½ Per Cent	Hemolytic Serum, in Units
		cc.			cc.	
1......	1	0.25	1.5	Add NaCl solution to make volume in each tube 1.5 cc. Incubate at 37–40° C. for 30 minutes	1.0	2
2......	1	.20	1.5		1.0	2
3......	1	.15	1.5		1.0	2
4......	1	.10	1.5		1.0	2
5......	1	.08	1.5		1.0	2
6......	1	.06	1.5		1.0	2
7......	1	.04	1.5		1.0	2
8......	1	.02	1.5		1.0	2
9......	1	.01	1.5		1.0	2
Controls	....	....	....		....	....
10......	1	....	....		1.0	2
11......	1	....	1.5		1.0	....
12......	1	....	1.5		1.0	2
13......	....	.2	1.5		1.0	2
14......	....	....	1.5		1.0	....
15......	....	0.2	1.5		1.0	....
16......	....	....	....		1.0	....

Add NaCl solution to bring final volume in each tube to 3.0 cc. Incubate at 37°–40° C. for 30 minutes. Examine for hemolysis and record results. Determine the antigenic unit.

From the titrations which have been performed, it is possible to determine the suitability of the reagents.

The anti-complementary dose is the smallest quantity of antigen which causes detectable inhibition of hemolysis.

An antigen which is found to be hemolytic of itself should not be used.

The antigenic unit is sometimes defined as the "smallest amount which gives complete inhibition in the presence of a unit of syphilitic serum." It is desirable, in the final Wassermann test, to use a unit 3–5 times as great because syphilitic sera may be encountered which are considerably weaker in reacting substance than those used in the standardization of antigen. *The antigenic unit is preferably defined as 3–5 times the smallest amount which gives complete inhibition in the presence of a unit of syphilitic serum.*

EXERCISE 5

1. **Wassermann test.**—After the units of amboceptor and antigen have been determined, the unit of complement titrated, and the patients' sera heated to 54°–56° C. for ½ hour on the day of the test, the test may be set up as in Table XV. It is convenient to use two rows of a water-bath rack—the back row for controls and the front row for tests. In Table XV the squares represent the arrangement of the tubes as seen by one looking down on the rack. Inside the squares appear the reagents listed in the order in which they are introduced and the amounts used. Incubate the preliminary mixture of reagents, lacking the elements of the hemolytic "indicator" system, in the water bath at 37°–40° C. for 1 hour. Then add the hemolytic sensitizer and erythrocytes where indicated, incubate in the water bath for ½ hour, examine the tubes for hemolysis, and record the results. Hold the tubes in the ice box or in a cool place (10°–15° C.) over night, re-examine, and record the results.

EXERCISE 6

1. **Flocculation tests for syphilis.**—When conducting the complement fixation test (Bordet-Gengou or Wassermann) ordinarily no visible precipitate forms nor is one usually detectable with the microscope. Examination with the ultra-microscope, however, reveals the definite aggregation of colloidal, submicroscopic particles. The principle of flocculation reactions applied as diagnostic tests for syphilis is the enhanced power of the serum to floccu-

TABLE XV

BACK ROW

Known Positive Serum	Known Negative Serum	Unknown Serum* No. 1	Serum No. 1 Hemolytic Control	Antigen Control	Hemolytic Control	Red Blood Cell Control	
cc. Serum 0.4 NaCl 1.6 Complement 1.0 Sensitized rbc. 1.0+	cc. Serum 0.4 NaCl 1.6 Complement 1.0 Sensitized rbc. 1.0+	cc. Serum 0.4 NaCl 1.6 Complement 1.0 Sensitized rbc. 1.0+	cc. Serum 0.4 NaCl 1.6 Complement 1.0 Red blood cells 1.0	cc. Antigen 2.0 Complement 1.0 Sensitized rbc. 1.0+	cc. NaCl 2.0 Complement 1.0 Sensitized rbc. 1.0+	cc. NaCl 3.0 Sensitized rbc. 1.0+	Other controls may be added

FRONT ROW

cc. Antigen 1.0 Serum 0.2 NaCl 0.8 Complement 1.0 Sensitized rbc. 1.0+	cc. Antigen 1.0 Serum 0.2 NaCl 0.8 Complement 1.0 Sensitized rbc. 1.0+	cc. Antigen 1.0 Serum 0.2 NaCl 0.8 Complement 1.0 Sensitized rbc. 1.0+

* Only one "unknown serum" and its control are shown in the table. Any convenient number of sera may be tested at the same time.

late colloidal matter. A number of modified Wassermann antigens have been developed (principally those modified by additions of tissue extracts, bile salts, lipoids, etc.). Cholesterinized antigens are most frequently used.

Sachs and Georgi described a cholesterinized alcoholic extract of heart muscle which, under suitable conditions, gives a specific flocculum with syphilitic serum. Dreyer and Ward have developed a similar test ("Sigma" reaction). Meinicke has developed three such tests. Kahn has developed a modified Sachs-Georgi test which is being widely tested in parallel with the Wassermann test. The advantages of flocculation tests over complement fixation tests as diagnostic and therapeutic guides still remain to be determined. When properly standardized, flocculation tests are more simply and more cheaply performed than corresponding tests based upon applications of the Bordet-Gengou phenomenon.

2. The Sachs-Georgi, Meinicke, Dreyer-Ward, Kahn, etc., flocculation reactions may be demonstrated by the instructor or by students especially interested in the technique of these tests. The details of the technique should be discussed with the class.

Kahn's technique for the flocculation test for syphilis[1] is given in detail.

I. GLASSWARE AND APPARATUS REQUIRED FOR THE TEST

All glassware employed in the test must be chemically clean.

1. **Test tubes.**—Standard antigen dilution tubes are 5.5 cm. in length and 1.5 cm. in diameter.

Standard tubes for performing the test are 7.5 cm. in length and 1 cm. in diameter.

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[1] Prepared from the *American Journal of Public Health,* XIV (1924), 498. For further details see R. L. Kahn, *Serum Diagnosis of Syphilis by Precipitation.* Baltimore, Md.: Williams & Wilkins Co., 1925. 237 pp.

2. **Test-tube racks.**—Racks should be of such construction as to permit vigorous shaking of the tubes.

3. **Pipettes.**—Ten cc. pipettes marked in 0.1 cc. quantities; 1 cc. pipettes marked in 0.01 cc. quantities; 0.2 cc. pipettes marked in 0.001 cc. quantities.

4. **Shaking machine.**—May be of any construction which will hold the test-tube racks employed.

5. **Inactivating bath** (56° C.) as well as centrifuge and centrifuge tubes may be of any make which will be found convenient in the particular laboratory.

II. REAGENTS EMPLOYED IN THE TEST

The three ingredients entering into the test are (1) antigen, (2) serum, and (3) physiologic salt solution.

1. ANTIGEN

Method of preparation.—The method of antigen preparation has recently been standardized with a view of eliminating several of the variable elements inherent in the method previously used.

The unit amount of powdered beef heart used for preparing antigen is 25 gm. and it is always extracted in a 250 cc. Erlenmeyer flask. The ether extraction consists of four ether "washings" of the powdered muscle at 10-minute intervals with 100, 75, 75, and 75 cc. ether, respectively. The subsequent alcohol extraction is carried out for three days at room temperature (21° C.). Twenty-five gm. of powdered beef heart will yield about 75 cc. antigen. If the preparation of larger amounts is desired, as many 25 gm. quantities are employed as needed.

Powdered heart muscle.—About 400 gm. of heart muscle are cut out from at least three fresh beef hearts and passed four times through a meat grinder. The ground material is spread into a thin layer on a porcelain platter or glass plate and dried by means of one or two revolving fans. After 6 or 8 hours, when the exposed surface is relatively dry, the material is turned over and drying continued over night. When the layer of beef heart is in the form of a dry plate, it is broken up into small pieces and drying continued until the material is brittle. The material is now ground into powder form by means of a mortar or coffee grinder which is used for no other purpose.

Powdered beef heart is now purchasable. It is prepared essentially as outlined above, except on a large scale—as many as seventy-five hearts entering into the preparation of a given lot. This assures a higher degree of uniformity than can be obtained with three beef hearts.

Extraction of powdered muscle with ether.—Twenty-five gm. of powdered beef heart are placed in a 250 cc. Erlenmeyer flask and 100 cc. ether (anesthesia) added. The flask is shaken from time to time for an interval of 10 minutes, after which the ether is filtered off. The filtration process may be hastened somewhat by applying gentle pressure to the beef heart by means of a spatula. Filtration is completed when pressure with the spatula does not cause drops of ether to pass through the funnel.

The moist beef heart is now returned to the original 250 cc. extraction flask. This may be done by first transferring the beef heart from the funnel to a sheet of white paper and breaking the material up with a spatula into pieces small enough for the mouth of the flask. Seventy-five cc. ether are now added and the mixture again shaken for a 10 minute interval and filtered as above.

After the second filtration of ether, the beef heart is transferred to the flask for a third time and 75 cc. ether again added. The mixture is shaken for a 10 minute interval and again filtered as described above.

The moist beef heart is now transferred for the fourth and last time to the flask and 75 cc. ether added, the mixture shaken for 10 minutes and filtered. After gentle pressure with a spatula does not cause drops of ether to pass through the funnel—the endpoint employed in each of the four ether filtrations—the beef heart is transferred to a sheet of white paper and dried either at room temperature or at 37° C. The drying usually requires from 10 to 15 minutes. When no ether odor is detectable, the beef heart is ready for extraction with alcohol. This extraction may be carried out in the same flask used for the ether extractions, provided the flask is entirely freed from ether odor.

Extraction of powdered muscle with alcohol.—The ether extraction being completed, the dry powdered muscle is weighed and placed in a 250 cc. flask. Usually there will be 23 gm. or less of the powder due to the loss during the ether extraction. Five cc. of 95 per cent alcohol are added per gm. of powder. The flask is shaken for 10 minutes and extraction allowed to continue at room temperature (21° C.) for three days without shaking. At the end of this period, the mixture is shaken for 5 minutes and filtered. The filtrate is kept in the dark at room temperature as stock antigen solution.

Cholesterinization of alcoholic extract.—A given amount of alcoholic extract—likely to be used in a month or two—is cholesterinized by adding 6 mg. of chemically pure cholesterin per cc. of extract. The cholesterin is dissolved by rotating the flask in a water bath at 37° C.

When all the cholesterin has been dissolved, the antigen is filtered to remove impurities and allowed to stand one day. It is then ready to be titrated or standardized for the test.

It is well to emphasize in connection with antigen preparation that the ether and alcohol employed should be of high purity and that the latter should be 95 per cent.

Tin-foil-covered corks have been found most satisfactory as stoppers for flasks used in antigen preparation and storing. Rubber as well as cork stoppers give off soluble elements into the alcohol which modify the final product.

Method of antigen titration.—The aim of the titration of antigen for this test is to find the minimum amount of physiologic salt solution to use with antigen which will result in an antigen-salt solution precipitate that is soluble on further addition of salt solution. The titration is carried out in the presence of salt solution and not in the presence of serum, although the latter may be used as a check on the titration, if desired.

One cc. amounts of cholesterinized antigen are added to each of five standard antigen dilution tubes (5.5 cm. length and 1.5 cm. diameter). To five similar tubes are added the following amounts of physiologic salt solution, respectively: 0.8, 0.9, 1.0, 1.1, and 1.2 cc. Each salt solution tube is emptied into a given antigen tube and, without waiting to drain the salt solution, the mixture is immediately poured back and forth five or six times to permit thorough mixing. Each of the five antigen dilutions will show the presence of a definite precipitate. The character of these precipitates, however, will vary according to the quantity of salt solution used. Some of the precipitates will be found to be stable and will not dissolve when mixed with salt solution; other precipitates will readily dissolve in salt solution.

The solubility in salt solution of each of the five antigen dilution precipitates is tested as follows: 0.05, 0.025, and 0.0125 cc. amounts, respectively, of each antigen dilution are pipetted with a 0.2 cc. pipette graduated in 0.001 cc. into three tubes (7.5 cm. in length and 1 cm. diameter). These small quantities are pipetted in each case to the bottom of the tubes, and 0.15 cc. quantities of physiologic salt solution are added to each tube. The rack is shaken vigorously for 2 minutes, after which 0.5 cc. salt solution is added to each tube and observation made as to whether or not the original antigen-dilution precipitate has redissolved. The antigen-dilution tube containing the smallest amount of salt solution in proportion to antigen, having a precipitate which redissolves in salt solution, as shown by this three-tube test, represents the endpoint of this titration and determines the proportion in which antigen is to be mixed with salt solution in the performance of the tests.

Table XVI gives an outline of a typical antigen titration.

TABLE XVI

Typical Antigen Titration for Test with Serum

Antigen dilution series	1	2	3	4	5
Antigen+salt solution, cc.	1+.8	1+.9	1+1.0	1+1.1	1+1.2
Result of dilution	Heavy precipitate in each antigen dilution				
Scheme used in testing solubility of precipitate in each antigen dilution	Tube no.		1	2	3
	Antigen dilution,* cc.		.05	.025	.0125
	Salt solution, cc.		.15	.15	.15
Solubility of precipitate as determined by three-tube test	Precipitate not soluble	Precipitate not soluble	Precipitate soluble	Precipitate soluble	Precipitate soluble
Standard antigen dilution			Antigen+minimum amount of salt solution giving precipitate which dissolves in salt solution		

* Each antigen dilution is allowed to stand 30 minutes after mixing antigen and salt solution before solubility test is made.

Tubes are shaken 2 minutes and 0.5 cc. salt solution added to each. All are observed for precipitates.

2. SERUM

The sera are separated from the clots by centrifugation in the usual manner, pipetted off and inactivated for 30 minutes at 56° C. The main point regarding sera is that they be free from red cells, fibrin, and particles of any kind. No difficulty is encountered in this test with milky or chylous sera or with moderately hemolyzed specimens. Such hemolyzed specimens as are not fit for a Wassermann test are likewise not fit for a precipitation test.

3. PHYSIOLOGIC SALT SOLUTION

Salt solution is prepared by dissolving 8.5 gm. of chemically pure sodium chloride per liter of distilled water and filtering. Although sterility of this solution is not essential, the same type of chemical cleanliness usually employed in quantitative chemical work is required.

III. THE ROUTINE TEST WITH SERUM

The routine test consists of three tubes containing three different proportions of serum and antigen dilution as shown in Table XVII.

TABLE XVII

Tube no.	1	2	3
Serum: antigen dilution	3:1	6:1	12:1
Antigen dilution, cc	.05	.025	.0125
Serum, cc	.15	.15	.15

Preparation of standard antigen dilution.—It is well when carrying out a number of tests to inactivate the sera and set up and number the tubes before diluting the antigen with salt solution for the tests. The antigen dilution is so standardized as to necessitate its use within a half-hour after mixing antigen with salt solution.

One cc. antigen diluted with 1 cc. salt solution gives sufficient antigen dilution for about eighteen tests. Two or 3 cc. antigen may be diluted with corresponding amounts of salt solution by utilizing the same standard antigen-dilution tubes.

Procedure: One cc. antigen is measured into an antigen-dilution tube. An amount of salt solution usually approximating that of the antigen, according to the titre of the antigen, is measured into a similar tube. The salt solution is poured into the antigen and, without waiting to drain the tube, the mixture is immediately poured back and forth five or six times to insure thorough mixing. For uniformity, this antigen dilution is permitted to stand 10 minutes at room temperature before pipetting.

As a preliminary control of the antigen dilution 0.05 cc. is measured into a test tube, 1 cc. salt solution added, and the tube shaken vigorously for 10 or 15 seconds. The mixture should appear opalescent.

Pipetting of antigen dilution.—The antigen dilution is always pipetted to the bottom of the tubes. The 0.05 cc. amounts may be pipetted with a 1 cc. pipette on which 0.05 cc. graduations are indicated with a wax pencil. For the 0.025 and 0.0125 cc. amounts of antigen dilution, 0.2 cc. pipettes are employed and the proper markings may also be indicated with a wax pencil. The antigen dilution should be mixed frequently during the pipetting period to assure a uniform mixture. Due to the possibility of evaporation of the small amounts of antigen dilution used in the test, it is necessary to pipette the serum within several minutes after the antigen dilution has been pipetted. If a worker desires to run forty precipitation tests, for example, it is better to pipette antigen dilution and sera for ten tests at a time than to pipette the antigen dilution for all the tests first and then follow with the sera.

Pipetting of serum.—The 0.15 cc. amounts of each serum are added to the antigen dilution by means of a 1 cc. pipette graduated in 0.01 cc. In pipetting these amounts of serum it is not necessary to lower the pipette to the bottom of the tube. As soon as the sera have been added for ten tests, or less, the rack is shaken sufficiently to insure thorough mixing of the serum with antigen dilution.

Shaking of tests.—After the sera have been mixed with the antigen dilution, the tests are shaken for a 2 minute interval. A shaking machine is of the utmost importance for this purpose, particularly in the examination of comparatively large numbers of specimens at a given time.

Effect of incubation.—Although the final results may be read immediately after the shaking period, different workers have observed that a 15 minute incubation period in the water bath at 37° C. produces sufficient clumping of the precipitates to make the reading of the results easier, particularly in the case of weak reactions. In studying the effect of 15 minute incubation on this test, no tendency for false positive reactions has been observed, and on the basis of easier reading the employment of this incubation period is of some advantage.

Addition of salt solution and reading of results.—After the shaking of the tests as well as after the 15 minute incubation period, if employed, the serum-antigen mixtures appear uniformly cloudy. In order to render the negative reactions clear and thus simplify the reading of results, 0.5 cc. salt solution is added to each tube. The tests should be read immediately after the addition of salt solution as an occasional weak precipitate may go back into solution on standing.

Readings are best made in front of a window with a darkened background. The negative sera appear opalescent and readily distinguishable without lifting the tubes from the racks. The strongly positive sera show heavy precipitates which are also easily read directly.

Only the tubes showing weak reactions need to be removed from the rack and examined individually. Each tube is lifted from the rack above the eye level, slanted to spread the fluid into a thin layer, and examined for a precipitate.

TABLE XVIII

Interpretation of Results of Routine Test with Serum

		1	2	3	Average of Reaction in the Three Tubes = Final Result
	Serum: antigen dilution	3:1	6:1	12:1	
	Antigen dilution, cc	.05	.025	.0125	
	Serum undiluted, cc	.15	.15	.15	
	Reaction No.				
Some Typical Reactions	1	++++	++++	++++	++++
	2	+++	++++	++++	
	3	++	++++	++++	+++
	4	+	++++	++++	
	5	–	++++	++++	
	6	–	+++	++++	++
	7	–	++	++++	
	8	–	+	++++	
	9	–	–	++++	+
	10	–	–	+++	
	11	–	+	++	
	12	–	±	++	±
	13	–	–	++	
	14	–	±	+	

Precipitation tests are read at night with the same ease as during the day. Light may be furnished by a 300-watt daylight bulb in an overhead indirect fixture.

Note.—If it is desired to make a check reading of the results at some later period, it is well to keep the tubes at ice-box temperature. Practically all positive reactions will be slightly stronger at the second reading. Occasionally, however, even at ice-box temperature, a serum which gave a –, +, ++ at the first reading may be found to be negative during the second reading. These reversible precipitation reactions have been observed especially in early primary and in highly treated cases. The reading made immediately after the addition of 0.5 cc. salt solution to each tube is the standard reading and is always the one reported.

CONTROL SYSTEM

a) **Antigen control.**—When pipetting antigen dilution for a series of tests, the last setup of three regular antigen amounts receives 0.15 cc. salt solution instead of serum and is read with the regular tests. All three tubes should show freedom from a precipitate.

b) **Serum controls.**—In the case of each positive reaction, the serum used in the test is examined for particles that might give the appearance of a specific precipitate.

c) **Positive and negative controls.**—One or more such controls are included with each series of tests.

INTERPRETATION OF RESULTS

A definite precipitate suspended in a clear medium is considered a complete reaction and is read 4+. Proportionally weaker reactions are read 3+, 2+, and 1+, respectively. The final result in each test is the average finding of the three tubes. Thus, if the precipitation reaction is 4+ in each of the three tubes, the final result is 4+. If the reaction is −, +++, ++++, the final result is 2+. A number of typical reactions with this method and the final result in each case are illustrated in Table XVIII.

EXERCISE 7

1. **Colloidal gold test (Lange's).**—In the absence of electrolytes, proteins precipitate colloidal suspensions; and in suitable concentrations, electrolytes produce the same effect. A colloidal suspension of a metal (i.e., gold) in a dilute salt solution is, however, stabilized or "protected" by a protein. Thus, Zsigmondy developed a method of measuring protein concentrations by their "gold numbers," i.e., the amount of protein necessary to protect from precipitation 10 cc. of a colloidal gold suspension of a particular concentration by 1 cc. of 10 per cent NaCl solution.

Lange discovered that, in spite of their abnormally high globulin contents, specimens of spinal fluid taken from patients suffering with syphilis of the central nervous system precipitated rather than protected colloidal gold. Hence came the development of Lange's test. Color changes and precipitation of gold plotted against dilutions of spinal fluid tested give the "gold curves." The troublesome feature of the test is the preparation of the gold solution.

2. **Preparation of reagents.**—(These should be prepared by the instructor.) All the glassware used in the test must be very carefully cleaned. It should be washed in hot water with pure soap; rinsed under running water, soaked (1 hr.) in hot bichromate cleaning solution; and then rinsed under running water, in distilled water, and finally in triply distilled water. (The water should be distilled from a hard-glass or quartz vessel to a quartz or block-tin condenser and stored in seasoned glassware or in a block-tin vessel, free from access of laboratory air.) Pure reagents should be used.

a) Heat 500 cc. of triply distilled water in a carefully cleaned beaker over a burner.

b) At 60° C. add 5 cc. of a 1 per cent solution of pure gold trichloride crystals in triply distilled water.

c) Place the beaker on a wire gauze and heat over a burner until the solution boils. Stir while heating.

d) When the solution begins to boil, remove the burner and add 5 cc. of a 2 per cent solution of potassium carbonate and 5 cc. of a 1 per cent solution of oxalic acid (both in triply distilled water). These reagents should be added rapidly.

The solution should have a red-orange color, should be perfectly clear, and should be neutral in reaction. Five cc. should be completely precipitated in 1 hour by 1.7 cc. of 1 per cent NaCl solution; should give a typical "paretic gold curve" with the spinal fluid from a paretic patient; and should not give more than a No. 1 test with spinal fluid from an individual probably free from syphilitic infection.

3. **Technique of the test.**—Arrange 11 thoroughly cleaned and dried test tubes in a rack. Using a freshly pre-

pared, sterile 0.4 per cent NaCl solution and a thoroughly cleaned and dried pipette, place 1.8 cc. of salt solution in tube 1 and 1.0 cc. in each of the other 10 tubes. With a clean, dry pipette, add 0.2 cc. of blood-free spinal fluid to the first tube and mix the reagents thoroughly. Remove 1 cc. from tube 1 and add to tube 2; mix thoroughly and transfer 1 cc. to tube 3; repeat the process through to the tenth tube. From this tube withdraw 1 cc. and discard.

TABLE XIX

Color Scale	Scale No.	Dilution of Spinal Fluid										Control
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
Colorless.	5											
Pale blue.	4											
Blue.	3											
Lilac or purple	2											
Red-blue.	1											
Red-orange. . .	0											

Tube 11 is a salt solution control and receives no spinal fluid. Add to each tube 5 cc. of colloidal gold solution and mix thoroughly. Make preliminary examinations after 30 minutes. Set the tubes aside (at room temperature) over night. They should be exposed to light only for a minimal period. Examine the next day and record the results numerically according to the following key:

0 = no change (by comparison with tube 11)
1 = reddish-blue
2 = lilac or purple
3 = blue
4 = pale blue (almost colorless)
5 = colorless; complete precipitation (and should be water clear)

The test should be conducted with a series of spinal fluids from cases in various clinical states (normal, paretic, luetic, meningitic). The results should be plotted as curves in a chart such as is shown in Table XIX.

SECTION XIV

PHAGOCYTOSIS

FOUR EXERCISES

EXERCISE 1

1. **Influence of hydrogen ion concentration on phagocytosis.**—The instructor will provide suspensions of guinea pig or rabbit leucocytes and suspensions of bacteria (i.e., *Staphylococcus aureus*) which have been adjusted to various hydrogen ion concentrations between $P_H = 6$ and $P_H = 9$. Mix in each of a series of tubes 0.1 cc. of bacterial and 0.2 cc. of leucocyte suspension, using suspensions of the same P_H. Incubate all tubes 30 minutes at 37°–40° C. in a water bath. Pour off the supernatant fluid from each tube. On separate slides make smear preparations from the sediments in the tubes. Let them dry at room temperature. Stain them by Gram's or by Jenner's method. Examine under the microscope; count the bacteria contained in 50 (or in 100) phagocytes on each. Calculate the phagocytic average. Write a brief discussion of the experiment.

EXERCISE 2

1. **Effect of normal and of immune serum on phagocytosis.**—The instructor will provide suspensions of rabbit leucocytes in sodium citrate—NaCl solution and of pneumococci in NaCl solution; some normal and some immune (anti-pneumococcus) horse or rabbit sera, each unheated and heated at 60° C. for 30 minutes. Prepare mixtures of the pneumococci with normal and immune sera, heated and unheated, and determine the phagocytic

averages for each as in Exercise 1. Record the results and write a discussion of them in the light of hypotheses on normal opsonin and immune opsonin (bacteriotropin).

EXERCISE 3

1. **Determination of opsonic index.**—The opsonic index is a ratio of two phagocytic averages: (1) the phagocytic average for leucocytes taking up bacteria in the presence of the patient's serum, (2) the phagocytic average for the same leucocytic and bacterial suspensions in the presence of normal serum (the pooled sera of 6 or more normal persons).

2. The student should use himself as the patient and 6 other students to provide the normal.

3. Prepare a Wright capsule or capillary pipette and collect about 10 drops of blood from your finger. Allow it to clot and the serum to separate. (Each 6 students should pool a little of their sera.) Collect 10–15 drops of blood from your finger in a little sodium citrate—sodium chloride solution in a centrifuge tube. Sediment the cells in the centrifuge and wash them once with salt solution. With a capillary pipette separate the upper layer of cells (phagocytes) (the "buffy coat").

4. With a clean capillary pipette, marked about 2 cm. from the tip, draw up 1 unit volume of your serum, a bubble of air, 1 volume of leucocyte suspension, a bubble of air, and 1 volume of bacteria. Mix by expelling into a watch glass and drawing into the pipette repeatedly. Seal the capillary end and incubate for 30 minutes at 37°–40° C.

5. Repeat the preparation with another pipette and watch glass, using the pooled sera of other persons instead of your own serum.

6. After incubating the mixtures, break the capillary tips, mix the materials again, prepare smears, dry and

stain them. Make phagocytic counts and calculate the opsonic index for your own serum.

EXERCISE 4

1. The nature of the union between opsonin, leucocyte, and bacterium.—Arrange the following mixtures in 2 tubes:

Tube	Cubic Centimeters
1	0.1 bacterial suspension
	.2 guinea pig serum
	0.7 salt solution
2	0.2 leucocyte suspension
	.2 guinea pig serum
	0.6 salt solution

2. Incubate the tubes in a water bath at 37°–40° C. for 30 minutes.

3. Centrifugate the tubes and wash the sediment once with salt solution.

4. Add to tube 1, 0.2 cc. leucocyte suspension, 0.3 cc salt solution; to tube 2, 0.1 cc. bacterial suspension, 0.4 cc. salt solution.

5. Incubate the tubes in the water bath for 30 minutes.

6. Prepare smears of sediment in each tube, stain them, and determine the phagocytic counts for each.

7. Write a discussion of the experiment.

SECTION XV

TOXINS AND ANTITOXINS

FIFTEEN EXERCISES

EXERCISE 1

1. **Preparation of diphtheria toxin.**—Incubate for five to ten days a culture of *Corynebacterium diphtheriae*, using a strain known to give good toxin production, in 100 cc. of sterile, neutral 2 per cent peptone broth in a 500 cc. flask. Filter through a tested Berkefeld filter into a sterile brown bottle. Store in the ice chest.

EXERCISE 2

1. **Determination of the minimal lethal dose (MLD) of a diphtheria toxin.**—(To reduce the number of guinea pigs used by a large class, the experiment should be performed by students in groups of 4 or 8.) The MLD is the smallest quantity which upon subcutaneous injection will regularly kill guinea pigs weighing 250 gm. each in four to five days. The MLD should first be determined approximately by injecting 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 cc. quantities of toxin subcutaneously into guinea pigs. Each quantity of toxin should be contained in a total volume of 2 cc. sodium chloride solution (0.8 per cent) should be used as the diluent.

2. After the preliminary titration of the MLD has been completed (this may be done by the instructor before the exercise) the toxin should be titrated precisely. For a satisfactory toxin, the preliminary titration should indicate an MLD of less than 0.01 cc. The precise titration is conducted by the technique given below. With a toxin of

approximate MLD of 0.005 cc., for example, inject into 250 gm. guinea pigs 2 cc. portions of salt solution containing the quantities of toxin shown in Table XX.

The material injected in each case should be carefully warmed at 37°–40° C. After each 2 cc. quantity has been injected, add 1 cc. of sterile salt solution to the syringe, preferably without removing the needle from the pig, and inject in order to remove from the syringe any appreciable portion of the toxin that may remain in it. The time of injection and of death of each animal should be recorded and the MLD estimated. Each guinea pig that dies should be autopsied. The typical gross lesions are local inflammation and necrosis at the site of injection and hyperemia of the suprarenals. The titration may be repeated and the MLD determined within limits narrower than 0.001 cc.

TABLE XX

Guinea Pig No.	Cubic Centimeters	
1	0.05	
2	.01	
3	.0075	
4	.007	
5	.006	
6	.006	
7	.005	
8	.005	
9	.004	
10	.004	
11	.003	
12	.003	
13	.05	(heated at 60° C. for 1 hr.)
14	0.0	(control)

EXERCISE 3

1. **Determination of the L_0 (Limes zero) and L_+ (Limes plus) doses of toxin.**—Prepare a quantity of accurately diluted standard diphtheria antitoxic serum with

sterile 0.8 per cent NaCl solution so that 1 cc. contains 1 unit of antitoxin. Using a toxin whose L_+ dose has been titrated in a preliminary manner and so diluted that the L_o quantity is contained in approximately 1.0 cc., prepare the mixtures shown in Table XXI. Inject the mixtures into a series of 250 gm. guinea pigs, using the technique given in Exercise 2, item 2. Record the time of injection and of death of each pig and the necropsy findings. Estimate the limits as follows:

TABLE XXI

Tube	Antitoxin (I Unit)	Toxin	0.8 Per Cent NaCl Solution
	cc.	cc.	cc.
1	1	0.9	1.1
2	1	1.0	1.0
3	1	1.1	0.9
4	1	1.2	.8
5	1	1.3	0.7
6	1	....	2.0
7	....	0.9	1.1

The L_o dose of toxin is the largest quantity which when injected subcutaneously with 1 unit of antitoxin into a guinea pig produces no local or systemic reactions of intoxication.

The L_+ dose of toxin is the smallest quantity which when injected subcutaneously into a 250 gm. guinea pig with 1 unit of antitoxin kills the animal in four to five days.

EXERCISE 4

1. **Titration of an antitoxic serum.**—A diphtheria antitoxic serum which has been titrated so that the approximate number of units per cc. are known should be provided.[1] The toxin whose L_+ dose has been determined

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[1] The instructor may suggest to advanced students experiments on the preparation, concentration, and purification of an antitoxic serum.

(Exercise 3) should be used for the antitoxin titration along the lines indicated here. Given, for example, an antitoxic serum which contains approximately 500 units per cc., dilute it 40 times with sterile 0.8 per cent NaCl solution, and arrange the mixtures shown in Table XXII. Mix in separate syringes 1 cc. of material from tubes 1–12, inclusive, with 2 cc. of toxin which has been diluted with sterile 0.8 per cent NaCl solution so that 1 L_+ dose (precisely) is contained in this volume. Three cc. from tube 13 is used without toxin. Hold the filled syringes at room temperature for 30 minutes. Then inject the contents into a series of 250 gm. guinea pigs. Record the time of each injection and the time of death of each animal. Estimate the number of units of antitoxin in the antitoxic serum.

TABLE XXII

Tube	Antitoxin (Diluted 1:40)	0.8 Per Cent NaCl Solution	Final Dilution of Antitoxin
	cc.	cc.	
1	1.0	9.0	1:400
2	1.0	10.0	1:440
3	1.0	11.0	1:480
4	1.0	12.0	1:520
5	1.0	13.0	1:560
6	1.0	14.0	1:600
7	1.0	15.0	1:640
8	1.0	16.0	1:680
9	1.0	17.0	1:720
Controls			
10	1.0		1: 40
11	1.0, heated 80° C., 15 min.		1: 40
12		2.0	
13		6.0	

The unit of antitoxin is the smallest quantity of antitoxic serum which will prevent the killing of a 250 gm. guinea pig by an L_+ dose of toxin.

EXERCISE 5

1. Titration of a toxin by the flocculation method.— The instructor should provide a standardized antitoxic serum (unconcentrated) and a toxin whose L_+ is known approximately. For an antitoxic serum containing, for example, 500 units per cc. and a toxin whose L_+ dose is approximately 0.4 cc., arrange the mixtures shown in Table XXIII in a series of tubes arranged in a rack in a water bath at 37°–40° C. After 15 and 30 minutes record the appearance of the mixtures, noting the development of cloudiness, and granular or flocculent precipitate. If no flocculent precipitate has formed after 30 minutes, the tubes should be kept under constant observation and a record made of the first appearance of flocculation in any one tube. The tube which shows the first flocculation is the I tube (indicator) and contains the neutral mixture. Other mixtures will show flocculation later. The student should calculate the L_f (Limes flocculation) for the toxin. *The L_f is the quantity of toxin which neutralizes 1 unit of antitoxin, measured by the flocculation method.* (It is recommended that the student read the paper by S. Bayne-Jones in the *Journal of Immunology*, IX [1924], 481.)

TABLE XXIII

Tube	Toxin	Antitoxic Serum
	cc.	cc.
1	2	(Quantities to be suggested by the instructor after preliminary tests)
2	2	
3	2	
4	2	
5	2	
6	2	
7	2	
8	2	
9	2	
10	2	

NOTE.—The instructor may prepare a comparatively simple apparatus for the precise measurements of small quantities of antitoxin: A tuberculin syringe is mounted in the vertical position on an upright board. Around the piston is looped a rubber band which can be fastened to a pair of staples inserted in the board above and to either side of the syringe. Above the piston is fastened the shaft and the micrometer head of a micrometer caliper. The shaft should be ground to a point which fits into a small hollow ground into the top of the piston. When syringe and micrometer are mounted in this manner the piston is supported by the contracting elastic and may be driven into the cylinder of the syringe by the point of the micrometer shaft when the head is turned. The apparatus should be calibrated gravimetrically so that the delivery from the needle is measurable in units on the micrometer head.

EXERCISE 6

1. **Schick test.**—This is a cutaneous test for immunity against the toxin of the diphtheria bacillus. Each group of students should perform the test on four white or albino guinea pigs prepared as follows: (*a*) normal; (*b*) injected intraperitoneally with 25–100 units of diphtheria antitoxin 1–12 hours previously; (*c*) injected with the same quantity of antitoxin previously heated at 75° C. for 1 hour; (*d*) injected with an immunizing mixture of toxin-antitoxin 3 to 5 times at five- to seven-day intervals. (The last injection should have been given one to three weeks before the animal is used in this experiment.)

Treat each of these animals in the following manner: (*a*) inject intracutaneously on the right side of the shaved abdomen 1/50 MLD of diphtheria toxin (contained in a volume of 0.1–0.25 cc.); (*b*) inject similarly on the left side the same quantity of the toxin previously heated at 75° C. for 15 minutes.

Examine the pigs twice a day for five days. Record the results and write a discussion of the experiment.

The students should be given the opportunity to see the Schick test performed on humans.

EXERCISE 7

1. **The titration of antitoxin in Schick-tested guinea pigs.**—The students (in conference with the instructor) should prepare the outline for an experiment to titrate the antitoxic content of the blood of each of the guinea pigs used in Exercise 6. The skin reactions of normal guinea pigs should be used as "indicator" for free toxin. The instructor should provide a toxin which has been titrated to determine the highest dilution (with 0.8 per cent NaCl solution) in which 0.1 cc. will produce necrosis or other reaction at the site of the injection when introduced intra-cutaneously into a large guinea pig. The experiment should be performed by the students working in groups.

EXERCISE 8

1. **Determination of the virulence and toxicity of diphtheria bacilli.**—Add to a culture on a slant of Löffler's medium or 2 per cent glycerin agar, 5 cc. of 0.8 per cent NaCl solution. Rotate the tube until the growth has been washed off the medium into an even suspension in the salt solution. Inject 1.0 cc. of the suspension subcutaneously into a normal guinea pig and 1.0 cc. into a guinea pig which has received 500–1,000 units of antitoxin. Keep the animals under observation for at least four days. If either dies, autopsy it and examine it for lesions typical of diphtheria intoxication and for infection in the guinea pig.

2. If a pure culture is not available, pick off diphtheria-like colonies from the culture under examination, emulsify it in salt solution, and inject as in item 1. A necropsy of a pig that dies is necessary to ascertain that the animal was killed by diphtheria bacilli or toxin. Cultures to Löffler's medium or glycerin agar should be made from the tissues near the site of the inoculation, the peritoneal fluid, and the heart blood.

3. Repeat the experiment, using for injections a distilled water suspension of diphtheria bacilli which have been washed at least 3 times with distilled water (by alternate centrifugation and resuspension in distilled water). The student should write a discussion of the experiment.

NOTE.—It is desirable that the instructor provide each two or three students with material from different strains of *Corynebacterium diphtheriae.*

EXERCISE 9

1. **Natural immunity of the rat to diphtheria toxin.**—The student should use diphtheria toxin whose MLD and L_+ dose are known (Exercises 2 and 3). Inject into each of two rats 1,000, 10,000, and 100,000 MLD of the toxin. On the following day record the lethality of these doses.

2. If the rats which received the smallest dose (1,000 MLD) are alive 24 hours later, bleed one; permit the blood to clot; separate the serum. (Retain the second rat to observe whether or not it dies later.) Inject the serum (5–10 cc.) intraperitoneally into a guinea pig. Record effect upon the animal.

3. Collect a few cc. of blood from a normal rat; defibrinate it and separate the plasma. Prepare the following mixtures: (*a*) 0.1 cc. of toxin diluted to contain 1/5 MLD + 0.9 cc. of 0.8 per cent NaCl solution; (*b*) 0.1 cc. of toxin (1/5 MLD) + 0.9 cc. rat plasma; (*c*) 0.1 cc. of salt solution + 0.9 cc. rat serum.

4. Incubate the mixtures in a water bath at 37°–40° C. for 30 minutes. Inject 0.1 cc. of each mixture intracutaneously (at three different sites) on the abdomen of a white guinea pig. Examine the animals daily. Record observations.

Write a discussion of the results.

EXERCISES 10 AND 11

1. **Tetanus toxin and antitoxin; tetanolysin.**—The instructor should provide standardized tetanus toxin and antitoxin. The unit for tetanus antitoxin is based upon a standard which is different from that used for diphtheria antitoxin. Dried, preserved toxin—instead of antitoxin—is used as the reference standard. The toxin unit is 100 MLD measured on guinea pigs weighing 350 gm. each. The unit of antitoxin is the quantity which completely neutralizes 10 such units of toxin (1,000 MLD).

2. The student should determine the tetanolysin titre of the tetanus toxin. The unit of tetanolysin may be taken as the smallest quantity which will give complete hemolysis with 1.0 cc. of a 2½ per cent suspension of washed rabbit erythrocytes in 0.8 per cent NaCl solution incubated in a water bath at 37°–40° C. for 1 hour.

3. After the tetanolytic unit has been determined, the student should determine the anti-tetanolytic unit of the antitoxin. This may be measured by determining the power of the antitoxin to prevent completely the hemolysis of 1.0 cc. of a 2½ per cent suspension of washed rabbit erythrocytes after incubation with 1 unit of tetanolysin at 37°–40° C. for 1 hour. The anti-tetanolytic titre of the antitoxin should be controlled by determining the titre of normal serum and of heated antitoxic serum because normal sera display considerable potency of this kind. The difference between the titres for the immune (antitoxic) and normal sera is a measure of the immune anti-tetanolysin.

4. **Tetanospasmin.**—Observe at frequent intervals each day for a week guinea pigs which have received the following materials: (*a*) 2–5 MLD of tetanus toxin (intramuscular injections); (*b*) a mixture of 5 MLD of toxin and 1 unit of antitoxin, incubated for 1 hour (intramuscular injection); (*c*) the supernatant fluid from a mixture of one-

half guinea pig cortex (ground) with 5 MLD of toxin: (1) The ground cortex and the toxin cooled to 0.5° C. before mixing and the mixture held at 0°–5° C. for 1 hour before the fluid is separated quickly in a cool centrifuge. (The separated fluid should be warmed to 37° C. immediately before the injection.) (2) Same as (1), except that the cortex and toxin are mixed and incubated at room temperature or at 37° C.

5. Write a discussion of the results of these experiments.

EXERCISE 12

1. **Preparation of botulinum toxin.**—Inoculate a culture of *Clostridium botulinum* (*Bacillus botulinus*) into a flask of clear, boiled beef heart medium while it is still warm and before it has absorbed an appreciable volume of air. The clear medium may be prepared by the following method:

> Infuse over night a mass of chopped beef heart with an equal weight of water. Strain off the fluid; heat it to boiling; adjust the P_H to 7.5; add peptone to give a 2 per cent concentration. Cover the extracted beef heart with water, boil for 10 minutes, and adjust the mixture to P_H 7.5. Pour off the fluid and wash the residue with water until the wash fluid is clear. Strain off the fluid from the residue through cheesecloth. Add the materials to tubes or flasks, using the proportions: $\frac{1}{4}$ meat, $\frac{3}{4}$ peptone broth. Plug with cotton and sterilize.

Seal the culture with sterile vaseline (1½ cm.) and incubate it at 37° C. for seven to ten days. Filter it through a Berkefeld filter. Preserve the filtrate in sealed tubes or ampules kept in the ice chest.

2. **Demonstration of the toxin.**—Inject intraperitoneally 0.1 cc. of the culture filtrate from item 1 into a mouse and 0.1 cc. into a guinea pig. Introduce with a pipette 1 cc. into the mouth of a guinea pig and force the animal to swallow the material. The instructor should intro-

duce 0.5 cc. of the filtrate into the stomach of a mouse (use a nasal sinus catheter). The animals should be held under observation for four days. Animals that die should be autopsied. No gross lesions should be observable.

3. **Preparation of botulinus antitoxin.**—The instructor may assign to students or may himself perform the preparation of the antitoxin in a sheep or goat.

4. **Demonstration of protective power and type specificity of antitoxin.**—The student should inject the following mixtures intraperitoneally into mice:

Mouse	
1 2	10 MLD botulinum toxin A
3 4	10 MLD botulinum toxin B
5 6	1 unit antitoxin A+10 MLD toxin A
7 8	1 unit antitoxin A+10 MLD toxin B
9 10	1 unit antitoxin B+10 MLD toxin A
11 12	1 unit antitoxin B+10 MLD toxin B

The mice should be kept under observation four days and autopsied.

Note.—The Hygienic Laboratory of the United States Public Health Service has established the following units for botulinum toxin and antitoxin: 1 MLD of butulinum toxin is the smallest quantity which will kill a 250 gm. guinea pig in 96 hours. With a particular preparation of dried toxin the Hygienic Laboratory established the unit of antitoxin. A quantity of the dried toxin which contained approximately 100 MLD was taken as the "test dose." The smallest quantity of a glycerinated anti-serum which when injected intraperitoneally with the "test dose" of toxin just permits the death of a 250 gm. guinea pig in

96 hours contains 1/10 of a unit of antitoxin. Hence, 1 unit of antitoxin is approximately equal in neutralizing potency to 990–1,000 MLD of the original dried toxin.

Although in the experiments described mice have been used, since they are more convenient and less expensive than guinea pigs for class work, the toxin and antitoxin units are those of the United States Public Health Service standards.

EXERCISE 13

1. **Preparation of the toxic products of dysentery bacilli.**—(*a*) Exotoxin: Add to a meat-infusion broth 10 per cent egg albumin. Adjust the P_H to 7.6–7.8. Sterilize. Inoculate with a culture of dysentery bacilli (Shiga) and incubate for five days at 37° C. Filter through a Berkefeld candle. (*b*) Endotoxin: Prepare cultures of dysentery bacilli on agar in bottles or Kolle flasks. Incubate for 24 hours at 37° C. Wash the growth off the agar with a small volume of 0.8 per cent NaCl solution. Incubate the suspension of bacteria at 37° C. for three days. Filter through a Berkefeld candle.

Divide each filtrate into two portions and heat one portion of each at 70° C. for 1 hour.

2. **Action of dysentery toxins.**—Of the exotoxin, inject 0.5 and 1.0 cc. quantities intravenously into young rabbits. Of the endotoxin, inject 2 and 5 cc. quantities intravenously into young rabbits. Keep the animals under observation for several days. Record the occurrence of muscular weakness, prostration, paralysis, loss of weight, diarrhea, etc. The animals that die should be autopsied and the peritoneum, intestines, brain, spinal cord, liver, and spleen should be examined macroscopically and microscopically for pathologic changes. Write a brief discussion on differences in the thermal stability and specific tissue toxicities of the exo- and endo-toxins.

EXERCISE 14

1. **Staphylolysin and anti-lysin.**—The student should be supplied with the filtrate from a virulent culture of *Staphylococcus aureus* and with the serum of a rabbit which had received repeated injections of such filtrates. The student should determine: (*a*) the lytic powers of the culture filtrate for washed and unwashed erythrocytes (human, horse, sheep, rabbit, guinea pig, etc.); (*b*) the thermal stability of these lytic properties; (*c*) the potency of the antiserum to inhibit lysis by staphylolysin; (*d*) the potencies of normal sera to inhibit lysis by the filtrate.

TABLE XXIV

Tube	2½ Per Cent Rbc. Suspension	Ricin	Serum	0.8 Per Cent NaCl Solution
	cc.	cc.	cc.	
1.........	1.0	0.1	....	1.9
2.........	1.0	0.5	....	1.5
3.........	1.0	1.0	....	1.0
4.........	1.0	0.1	1.0	0.9
5.........	1.0	0.5	1.0	0.5
6.........	1.0	1.0	1.0	....
7.........	1.0	....	....	2.0
8.........	1.0	....	1.0	1.0
Controls	....	....	....	....
9.........	1.0	....	1.0*	1.0
10.........	1.0	....	....	2.0

* One-half saturated $ZnSO_4$ solution in H_2O.

EXERCISE 15

1. **Hemagglutination by ricin, *in vitro*.**—Set up the mixtures shown in Table XXIV, using a solution of ricin (1 pt. in 1,000 pt. 0.8 per cent NaCl solution) and using erythrocytes and serum from guinea pigs and sheep. Some students should use the guinea pig and others the sheep reagents.

2. **Hemagglutination by ricin, *in vivo*.**—Inject 25 cc. of the ricin solution intraperitoneally into a guinea pig. Bleed from the heart 30 and 60 minutes and 2 hours after the injection. Examine the blood immediately for hemagglutination, crenation of the erythrocytes, etc., using direct microscopic examination of a drop between a slide and a cover glass. Retain the animal until it dies in a cage equipped for the collection of the urine. Examine the urine for hemoglobin color and crystals.

SECTION XVI

INVASIVENESS; ANTI-BACTERIAL SERA; MISCELLANEOUS

EIGHT EXERCISES

EXERCISE 1

1. **Invasiveness.**—(Half the working groups in the class may be assigned experiment [*a*] and half [*b*].) (*a*) Inject intraperitoneally into each of 8 mice a few anthrax bacilli taken from a virulent culture. At 12 hour intervals kill a mouse and study smears and subcultures from the heart blood, liver, and spleen for the approximate number of anthrax bacilli present. Prepare sections from the liver and spleen. Determine whether there is any correlation between numbers of bacteria in the blood and in the organs. Discuss the virulence of *Bacillus anthracis* for the white mouse. (*b*) Collect a sample of blood from a rat heavily infected with *Trypanosoma lewisi*. Obtain the blood by snipping the tip of the tail. Determine with the hemocytometer cell or by the method given in Section XI, Exercise 1, the approximate number of parasites in the blood. Etherize the rat; open the chest; and, with a hypodermic needle and syringe, collect 6 cc. of blood from the heart. Mix the blood promptly with 25 cc. of cool 0.7 per cent NaCl solution. Determine the number of trypanosomes per cc. Prepare dilutions to contain 100,000,000, 1,000,000, 10,000, and 10 per cc. Inject 1 cc. quantities of these dilutions intraperitoneally into a series of 4 mice. Determine, by examination of blood from each mouse 12, 18, 24, 36, 48, 96, 120, etc., hours later, whether there is

any correlation between the number of trypanosomes injected, the time of appearance, and the concentration of the trypanosomes in the blood. Record the time of death of each mouse. Write a discussion of the results.

In place of *T. lewisi* the following may be substituted: *T. equinum, T. equiperdum, T. brucei, T. evansi,* or *T. hippicum.*

EXERCISES 2 AND 3

1. **The typing of pneumococci.**—(The instructor will determine whether the method of Krumwiede and Valentine,[1] of Avery,[2] of Sharp,[3] etc., will be used.)

2. **Determination of the virulence of pneumococci for white mice.**—Each group of students should make the determination for a culture of pneumococci of known type. Have all types represented in the work of the class. Use the following technique: (*a*) Prepare a 24 hour culture on a blood agar slant. (*b*) Pour 10 cc. of Blake's broth[4] over the slant and incubate for 5 hours. (*c*) Transfer 0.5 cc. of the broth suspension to a tube containing 10 cc. of Blake's broth and incubate the inoculated tube at 37° C. for from 16 to 18 hours. (*d*) Prepare decimal dilutions of the culture in sterile broth so that quantities of the culture from 0.1 to 0.000,000,01 cc. are contained in a volume of 0.5 cc. (*e*) Warm the broth dilutions to 37° C. and inject 0.5 cc. of each intraperitoneally into each of 2 mice. (*f*) Inject into each of 2–4 mice 0.5 cc. of sterile dilution broth to serve as

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[1] See Park, Williams, and Krumwiede, *Pathogenic Microorganisms.*

[2] *Journal of the American Medical Association,* LXX (1918), 17.

[3] *Journal of Infectious Diseases,* XXXVII (1925), 473.

[4] Blake's broth is prepared by the following method: Infuse 1 lb. of lean chopped beef with 1 liter of tap water in the ice chest over night. Boil the infusion for 30 minutes, filter it through paper, and add water to restore the loss by evaporation. Add 1 per cent peptone (Fairchild) and 0.2 per cent anhydrous Na_2HPO_4. Boil for 20 minutes. Adjust the P_H to 8.0. Sterilize in the Arnold sterilizer for 20 minutes on each of three successive days. The final P_H should be 7.8.

controls on the technique. (*g*) Keep the mice under observation for 48 hours. Record the time of death of each.

3. Correlation and discussion with instructor of the findings of the class.

EXERCISES 4 AND 5

1. Determine the agglutinating and precipitating titres of a set of anti-sera for pneumococci and for culture filtrates from them, respectively. The instructor should provide mono- and poly-valent anti-pneumococcus sera of at least two varieties—horse anti-serum, antibody solution, etc.

2. **The protective action of pneumococcus anti-sera.**—The instructor should provide each working group with a normal and a mono- or poly-valent anti-serum and a culture of pneumococci. Having determined the minimum fatal dose of pneumococci for the white mouse (see Exercises 2 and 3, item 2), inject intraperitoneally into a series of mice mixtures containing a constant quantity of serum (i.e., 0.1 or 0.5 cc.) and various quantities of pneumococci ranging from 1 to 1,000,000,000 minimum fatal doses, each quantity contained in 0.5 cc. of fluid. The cultures of pneumococci should be prepared by the technique described in Exercises 2 and 3, item 2. From the mortality of the mice in the subsequent 48 hours, estimate the protective properties of the serum used in terms of protection against minimum fatal doses of pneumococci. For added accuracy, the controls in this experiment should be the mortality of mice injected with the various doses of pneumococci mixed with the constant quantity of heated anti-serum as well as with normal serum.

3. The instructor may designate one or two groups in the class to demonstrate the protective action of an anti-pneumococcus serum against intra-tracheal injections of pneumococci into rabbits. Ten, 1,000, and 1,000,000 mini-

mum fatal doses for mice may be injected into normal rabbits and into rabbits that have received, intravenously, 10–30 cc. of anti-serum. Rectal temperatures and leucocyte counts should be made for each animal before the experiment and at frequent intervals thereafter. The animals that die should be carefully autopsied.

EXERCISE 6

1. The student should arrange the protocols for experiments to determine the effects of a type-specific, pneumococcus anti-serum upon the phagocytosis of pneumococci in the peritoneal cavity and in the blood stream of the white mouse.
2. After approval of the protocol by the instructor the student should perform the experiment.

EXERCISE 7

1. **Disappearance of injected bacteria or other cells from the blood stream.**—The student should arrange the protocol of an experiment to determine the rate of disappearance of injected bacteria from the circulating blood of normal and immunized animals. It is desirable to use in this experiment pure cultures of such bacteria as can be easily detected in the blood by microscopic examination and can be easily counted for viable numbers by plate counts. The rabbit is a convenient animal for such an experiment.
2. At the discretion of the instructor, the bacteria suggested as antigen in this experiment may be replaced by erythrocytes and the hemolytic rather than "bactericidal" action studied *in vivo*.
3. The student should perform the experiment after the protocol has been approved by the instructor.

EXERCISE 8

1. **Immunity due to inhibition of reproduction of parasites.**—This is a type of immunity which appears to be

dependent upon the inhibition of the reproduction of the parasites but not upon their destruction.[1] Inject 12 large rats intraperitoneally with *Trypanosoma lewisi*. Make blood slides daily for ten days. During this period the parasites undergo a reproduction cycle, and by the tenth day practically all of the parasites have become non-reproducing adults. This should be noted in the blood smears. Hold the animals until about the twentieth day after injection because the titre of reproduction inhibiting immune body increases steadily. At this time the blood contains large numbers of "adult," non-reproducing parasites as well as the immune body.

2. Bleed the rats to death and remove the red blood cells by slow centrifugation. The trypanosomes remain in the supernatant serum. These are removed by rapid centrifugation of the separated serum. Save the trypanosome-free serum. Wash the sedimented trypanosomes with cool 0.7 per cent NaCl solution once or twice. For control tests, bleed normal, uninfected rats and separate the serum. (Blood samples from these rats should be examined to determine that they are not infected.)

3. Inject 12 small (30–50 gm.) rats intravenously in the tail vein each with 0.5 cc. of trypanosome suspension. Inject 6 of these rats intraperitoneally with the following doses of the immune serum and 6 more with similar doses of normal serum:

1............ 0.25 cc. per 100 gm. of body weight
2............ .50 cc. per 100 gm. of body weight
3............ 0.75 cc. per 100 gm. of body weight
4............ 1.00 cc. per 100 gm. of body weight
5............ 2.00 cc. per 100 gm. of body weight
6............ 3.00 cc. per 100 gm. of body weight

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[1] See W. H. Taliaferro, *Quarterly Review of Biology* (1926), No. 2. Also W. H. Taliaferro, *Journal of Experimental Medicine*, XXXIX (1924), 171–90; F. A. Coventry, *American Journal of Hygiene*, V (1925), 127–44.

Make blood slides daily from each rat for two weeks. Stain by Wright's method. Examine microscopically for evidences of division and reproduction of the trypanosomes. It is not essential to find dividing parasites. The non-reproducing adults are uniform in size, whereas reproducing forms are highly variable.

NOTE.—This "reaction product" immunity is demonstrable with the non-lethal but not with the lethal trypanosomes.

The student should write a discussion of the results in this exercise.

SECTION XVII

HYPERSENSITIVITY: ANAPHYLAXIS; ANAPHYLATOXIN; TUBERCULIN REACTIONS

SIX EXERCISES

EXERCISES 1 AND 2

1. **Acute anaphylaxis in the guinea pig.**—Three weeks before the anaphylactic state is to be demonstrated in the guinea pigs, the student or instructor should sensitize a series of animals by intraperitoneal or intravenous injections as follows:

SENSITIZATION

Animal No.	
1 2 3	0.005 gm. egg albumin in 2 cc. of 0.8 per cent NaCl solution
4 5 6	0.25 cc. horse serum in 2 cc. of salt solution
7 8 9	0.25 cc. sheep serum in 2 cc. of salt solution

2. To demonstrate anaphylaxis, specificity, and desensitization, the animals listed in item 1 should be used in the following manner:

INTOXICATION

Animal No.

1. Inject intraperitoneally or (preferably) intravenously 0.050 gm. egg albumin contained in 2 cc. of salt solution. Observe for the signs of anaphylaxis in the guinea pig
2. Before the exercise, inject subcutaneously three times at 1- or 2-hour intervals 0.0005–0.001 gm. egg albumin in 0.1 cc. of salt

solution. Observe the reactions (local and systemic) to these desensitizing injections. If the animal survives, inject it as indicated for No. 1

4 and 7 } Inject intravenously 0.5 cc. horse serum. Observe for anaphylaxis

5 and 8 } Inject intravenously 0.5 cc. sheep serum. Observe for anaphylaxis

3, 6, and 9 } Save for Exercise 3

If any of the animals which show acute anaphylaxis survive, reinject them in the same manner after the effects of the first shock have disappeared. Note the results.

EXERCISE 3

1. **Anaphylaxis *in vitro*** (isolated uterine strip).—The instructor or a group of advanced students should remove the uterus from guinea pigs Nos. 3, 6, and 9 sensitized in Exercises 1 and 2, divide each uterus into two parts and, when each is to be tested, mount each part on an apparatus arranged after the Schultz-Dale method to obtain a kymographic record of the contractions. The uteri should be kept in warm, neutralized Ringer-Locke solution (P_H 7.4) when not being used. After a tracing has been obtained of the normal contractions of a strip, it may be tested for sensitivity to reagents in the order suggested in the following protocol:

Animal No.

3 { Horn *a:* egg albumin; egg albumin; sheep serum
Horn *b:* sheep serum; horse serum; egg albumin

6 { Horn *a:* horse serum; horse serum; sheep serum; egg albumin
Horn *b:* egg albumin; sheep serum; horse serum

9 { Horn *a:* sheep serum; sheep serum; horse serum
Horn *b:* egg albumin; horse serum; sheep serum

EXERCISE 4

1. **Passive anaphylactic sensitization of guinea pig.**—Inject intraperitoneally into each of 2 guinea pigs 5.0 cc. of serum from a rabbit which has been sensitized to egg albumin three to five weeks previously. Twenty-four or 48 hours later inject intravenously into each of these treated guinea pigs and into a normal guinea pig 0.005–0.010 gm. egg albumin contained in 1.0 cc. of 0.8 per cent NaCl solution warmed to 38° C. Observe for anaphylaxis.

2. **Precipitins and anaphylaxis.**—The instructor should demonstrate anaphylactic shock in rabbits which have been sensitized to egg albumin and to horse or sheep serum, respectively. In addition, a normal rabbit should be injected with each antigen. The students should bleed these rabbits before and after they are injected with antigen, separate the serum from each, and test these sera for precipitins against the homologous and heterologous antigens. If acute anaphylactic shock is observed in one of the rabbits, immediately after its death the instructor should open its chest and demonstrate the collapsed condition of the lung.

EXERCISE 5

1. **Cutaneous anaphylaxis.**—Shave the abdomen of a sensitized white rabbit four to six weeks after the injection of the last sensitizing dose of antigen. Make two superficial abrasions in the skin. Into one rub gently a little of the specific antigen; into the other a little 0.8 per cent NaCl solution. Observe for local reactions. Some hours later inject a small quantity of antigen[1] subcutaneously. Keep the animal under observation for local and systemic reactions during the succeeding 24 hours.

2. **Anaphylatoxin type of reaction.**—Inject intravenously into a guinea pig 3–6 cc. of melted 0.5 per cent

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[1] For example, if the antigen is a serum, 0.005–0.01 cc. will suffice.

solution of agar in 0.8 per cent NaCl solution cooled to 42° C. Observe reactions. Write a discussion of the results of these experiments.

EXERCISE 6

1. **Cutaneous tuberculin reaction.**—The instructor will provide normal guinea pigs and guinea pigs which have been rendered tuberculous by injection of tubercle bacilli four to six weeks earlier. The student will inject 0.05, 0.075, and 0.10 cc. quantities of tuberculin intracutaneously in the plucked and cleansed skin of the abdomen of a normal and of a tuberculous animal. The guinea pigs should be examined some 6, 12, 24, and 48 hours later and at further intervals until there has been subsidence of any reactions at the sites of the injections.

2. **Testicular tuberculin reaction.**—Inject into the left testis of a guinea pig that was injected with tubercle bacilli one month to six weeks previously 0.1 cc. of tuberculin (1:1 dilution of BE or 1:10 dilution of OT tuberculin). One month after the tuberculin injections were made, the animal should be killed and sections prepared from the left (injected) and right (control) testes. When feasible, a series of guinea pigs should be injected in the manner indicated, each with a different dose of tuberculin and the unit of tuberculin determined as measured by the spermatocyte reaction.[1]

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[1] The student should refer to E. R. Long, *Journal of Infectious Diseases*, XXXVII (1925), 368, and F. W. Stewart, *Journal of Immunology*, XI (1926), 123.

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